

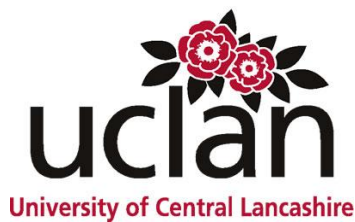
Pharmacokinetics and pharmacodynamics of some NSAIDs in horses: A pharmacological, biochemical and forensic study

By

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degree of Doctor of Philosophy**

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Dedicated to:

H.H. Sheikh Mohammad Bin Rashid Al Maktoum

Prime Minister and Ruler of Dubai, UAE.

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have been in use for over 100 years to treat pain, exerting their analgesic effect by inhibiting prostaglandin (PG) synthesis via the COX pathway. Some of the NSAIDs have adverse side effects including ulceration of the stomach and cardiovascular events which are associated with bleeding. Search is still going on to find a safe NSAID. Two new coxib NSAIDs, namely celecoxib and etoricoxib have been developed and they exert marked beneficial effects in reducing pain in humans and other small animals with little or no side effects. No such study has been done on horses to see if they can tolerate the drug as an analgesic pain killer. This study was designed to investigate the effects of the two coxib NSAIDs, celecoxib and etoricoxib in six retired race horses to determine any adverse side effects of the drugs, the time course changes in their metabolism and elimination once administered orally in known physiological doses and the metabolites produced by each drug over time. The study employed well established clinical and biochemical techniques to measure blood-borne parameters and the metabolism of each drug. The results show that either etoricoxib or celecoxib had no adverse side effects on blood borne parameters and the stomach of the horses. Pharmacokinetic study following oral administration of 2 mg/kg b wt of either celecoxib or etoricoxib to the six race horses showed a C_{max} of $1.15 \pm 0.3 \mu\text{g/ml}$, t_{max} , to be $4.09 \pm 1.60 \text{ hr}$ and a terminal half-life of $15.52 \pm 1.99 \text{ hr}$ for celecoxib and a C_{max} of $1.0 \pm 0.09 \mu\text{g/ml}$, t_{max} of $0.79 \pm 0.1 \text{ hr}$ and, terminal half-life of $11.51 \pm 1.56 \text{ hr}$, respectively for etoricoxib. The results also show that each coxib is metabolized in the horse and both the parent drug and its metabolites are found in the urine, plasma and faeces. The results have also shown that even small traces of either drug or its metabolites can be measured in urine samples even 120 hours following oral administration. The main metabolites found in plasma, urine and faeces are hydroxyl celecoxib and carboxycelecoxib when celecoxib was administered orally to the 6 retired race horses. Similarly, hydroxymethyletoricoxib, carboxylic etoricoxib, hydroxymethyl-1-N-oxide metabolite of etoricoxib and hydroxymethyletoricoxib glucuronide were also found in plasma, urine and faeces following oral administration etoricoxib to the animals. The results for either horse hepatocytes or camel liver show to some extent similar metabolites. In conclusion, the results show that both drugs have no adverse side effects in the horse and their metabolites are completely eliminated within 120 hours following oral administration.

Index

<u>Chapter I : General Introduction</u>	1
1.1 Historical perspective of Horse Racing	2
1.2 Inflammation and drug treatment	3
1.3 Treatment of pain	3
1.4 Different Chemical Groups of NSAIDs	4
1.5 Mechanism of Action of NSAIDs	5
1.6 Cyclooxygenase isoform, structure and function	9
1.7 Cyclooxygenase-1: the constitutive isoform	11
1.8 Cyclooxygenase-2: the inducible form	13
1.9 Some common NSAIDs used to treat horses	16
1.9.1 Phenylbutazone	19
1.9.2 Flunixin	20
1.9.3 Naproxen	22
1.9.4 Ketorolac	22
1.9.5 Ketoprofen	23
1.9.6 Carprofen	23
1.9.7 Tramadol	24
1.9.8 Meloxicam	24
1.9.9 Firocoxib	25

1.10	New COX-2 Inhibitors	27
1.10.1	Pharmacokinetics and Pharmacodynamics of celecoxib and etoricoxib	28
1.10.2	Pharmacokinetics and pharmacodynamics of celecoxib	29
1.10.3	Pharmacokinetics and pharmacodynamics of etoricoxib	34
1.10.4	Method of Analysis of Celecoxib	37
1.10.5	Methods of Analysis of etoricoxib	38
1.11	Structure and function of the GI tract and the kidney in drug metabolism	41
1.12	The metabolism and fate of drugs in the body	44
1.13	Liver enzymes and drug metabolism	47
1.14	Cytochrome P450 enzymes in horses	49
1.15	Metabolite profiling and identification <i>in vitro</i>	50
1.16	Gastrointestinal safety of COX-1 and COX-2 inhibitors in horses	51
1.17	Liquid chromatography-Mass spectrometry	54
1.18	Mass Spectrometry	54
1.19	Triple Quadrupole Mass Spectrometry	55
1.20	Working hypothesis	60
1.21	Main Aims	60
1.22	Specific Aims	60

<u>Chapter II: Materials and method</u>	62
2.1 Materials	63
2.2 Methods	64
2.2.1 Animals	64
2.2.2 Drug material	65
2.2.3 Treatment group and study protocol	65
2.2.4 Drug administration and sample collection	65
2.3 Measurements of blood-borne biochemical and anion and cation parameters following oral administration of either etoricoxib or celecoxib	67
2.4 Optimization of the method and extraction of etoricoxib from plasma	69
2.5 Preparation of etoricoxib stock solution and standards for plasma analysis	69
2.6 Extraction of etoricoxib from plasma samples	70
2.7 Etoricoxib extraction recovery, assay precision and accuracy	71
2.8 Extraction of etoricoxib and metabolites from urine samples	71
2.9 Extraction of etoricoxib from faeces	73
2.10 Determination of etoricoxib concentration	73
2.11 Assay procedure for celecoxib	74
2.12 Preparation of stock solution of celecoxib and internal standard	74
2.13 Extraction of celecoxib from plasma samples	75
2.14 Celecoxib extraction recovery, assay precision and accuracy	75
2.15 Extraction of urine and faeces for celecoxib metabolite profiling	76
2.16 LC-MS-MS conditions for celecoxib administration samples	77

2.17	Pharmacokinetic Methods	77
2.18	In vitro drug metabolism using either commercial horse haepatocytes or isolated camel liver fragments	78
2.19	Metabolic study using fresh camel liver	78
2.20	Statistical analysis of data	79

Chapter III : Animal characteristics, haematological and biochemical factors in blood following oral administration of either etoricoxib of celecoxib

3.1	Introduction	81
3.2	Methods	81
3.3	Results	82
3.3.1	Animal characteristics	82
3.4	Measurement of haematological and biochemical parameters	82
3.5	Discussion	92

Chapter IV: Pharmacokinetics and metabolism of celecoxib and etoricoxib in horses

4.1	Introduction	102
4.2	Method	102
4.3	Results	103
4.3.1	Optimisation of extraction method for celecoxib and etoricoxib	103
4.3.2	Celecoxib analytical method validation	105

4.4	Pharmacokinetics of celecoxib	106
4.5	Metabolism and excretion of celecoxib in plasma, urine and faeces	108
4.6	Identification of COOH-celecoxib and OH-methyl celecoxib	114
4.7	Etoricoxib analytical method validation	122
4.8	Pharmacokinetics of etoricoxib	123
4.9	Metabolism and excretion of etoricoxib in plasma, urine and faeces	126
4.10	Identification of hydroxymethyl, carboxylic acid and hydroxymethyl- etoricoxib-1'-N-oxide metabolite of etoricoxib	134

<u>Chapter V: Metabolic profiling of celecoxib and etoricoxib using liver microsomes and tissues</u>	150
---	------------

5.1	Introduction	151
5.2	Method	152
5.3	Result	152
5.3.1	Metabolism of etoricoxib by horse liver microsomes	152
5.3.2	Metabolism of celecoxib by horse liver microsomes	153
5.3.3	Metabolism of etoricoxib and celecoxib by camel liver tissue	156
5.4	Discussion	161
5.4.1	Metabolism of etoricoxib	164
5.4.2	Metabolism of celecoxib	165

<u>Chapter VI: General Discussion</u>	168
--	------------

6.1	Introduction	169
6.2	Measurements of haematological parameters	170

6.3	Some adverse side effects of NSAIDs	173
6.4	Pharmacokinetics and Pharmacodynamics of some common NSAIDs in horses	175
6.5	Metabolism and pharmacokinetics of celecoxib and etoricoxib <i>in vivo</i>	178
6.6	Metabolism of celecoxib and etoricoxib <i>in vitro</i>	182
6.7	Concluding remarks	185
<u>Chapter VII: Conclusion and Scope for future studies</u>		186
7.1	Conclusions	187
7.2	Scope for future studies	188
<u>Chapter VIII: References</u>		191
<u>Appendices</u>		227

List of figures

Chapter-I: General Introduction

Figure 1.1	The arachidonic acid cascade	10
Figure 1.2	Structure of COX-1 and COX-2	12
Figure 1.3	Schematic representation of the inhibition of COX-1, Inhibition of COX-2 by a nonselective NSAID and inhibition of COX-2 by COX-2 selective NSAID.	12
Figure 1.4	The Cyclo-oxygenase pathway	15
Figure 1.5	Chemical structures and evolution of major classes of NSAIDs prior to discovery of COX-2	18
Figure 1.6	Chemical structures of some commonly used NSAIDs in horses to treat pain	21
Figure 1.7	Chemical structures of the coxib NSAIDs	26
Figure.1.8	Metabolic pathway for celecoxib in humans	40
Figure 1.9	Metabolic pathways of etoricoxib in healthy human volunteers	43
Figure 1.10	Schematic diagram of a triple stage quadrupole mass analyzer	58
Figure.1.11	Schematic description of different scan modes that can be use in a triple quadrupole mass spectrometer	59

Chapter –II: Materials and method

Figure.2.1	Photograph showing the race horses housed in stables used in the study	66
Figure 2.2	Photograph showing the THERMO FINNIGAN TSQ ACCESS LC/MS/MS	72

Chapter III: Animal characteristics, haematological and biochemical factors in blood following oral administration of either etoricoxib and celecoxib

Figure 3.1	Original endoscopic photographs showing the mucosa of the stomach of untreated horses prior to administration of either 2 mg/kg b wt etoricoxib (A) or 2 mg/kg b wt celecoxib (B) and following treatment for 15 days with either etoricoxib (C) or celecoxib (D).	85
Figure 3.2	Picture showing the presence of extensive deep ulcer after continuous drug administration	86
Figure 3.3	Bar charts showing the blood concentrations of RBC ($10^{12}/L$), PCV (L/L), EOS (%), MONO (%), Hb (G/dL), BASO (%) in pre admin and post administration of etoricoxib (2 mg/kg b wt) and celecoxib (2 mg/kg b wt) in horses	87
Figure 3.4	Bar chart showing the plasma concentrations of WBC ($10^9/L$), MCH (pg), MCHC (g/dL), LYM (%), MCV (fL), NEU (%), PLT ($10^9/L$) in pre admin and post administration of etoricoxib (2 mg/kg b wt) and celecoxib (2 mg/kg b wt) in horses.	88
Figure 3.5	Bar charts showing the plasma concentrations of CK (U/L), LDH (U/L), AST (U/L), ALT (U/L), GGT (U/L), CREA ($\mu\text{mol/l}$), BUN (mmol/l), TP (g/l) and ALB (g/l) in pre administration and post administration of etoricoxib (2 mg/kg b wt) and celecoxib (2 mg/kg b wt) in 6 horses.	89
Figure 3.6	Bar charts showing the plasma sample concentrations of Ca^{2+} (mmol/l), PHOS (mmol/l), Na^{+} (mmol/l), K^{+} (mmol/l), Cl^{-} (mmol/l), Cu^{2+} ($\mu\text{mol/l}$), Zn^{2+} ($\mu\text{mol/l}$) and SAA ($\mu\text{g/ml}$), in pre administration and post administration of 2 mg/kg b wt etoricoxib and 2 mg/kg b wt celecoxib in 6 horses.	90
Figure 3.7 (A)	Time course changes in the plasma levels of creatinine either before (0 hour) or after the oral administration of either 2 mg/kg b wt etoricoxib or 2 mg/kg b wt celecoxib in horses.	91
Figure 3.7 (B)	Bar charts showing the plasma levels of creatinine either before or after administration of either celecoxib or etoricoxib.	91

Chapter –IV: Pharmacokinetics and metabolism of celecoxib and etoricoxib in horses

Figure 4.1	Original chart recordings of chromatograms showing (A) the retention time of zaleplon (IM), (B) the absence of any signal at the RT of celecoxib, (C) the peak for zaleplon (IS) and (D) the peak for celecoxib spiked in blank plasma.	104
Figure 4.2	Time course changes (mean \pm S.E.M) of plasma concentration of celecoxib following oral administration of a single dose of 2 mg/kg b wt to 6 healthy horses.	109
Figure 4.3	Time course changes in celecoxib excretion in (A) urine samples of 6 horses employed in this study and (B) the mean (\pm SEM) data for the six animals.	110
Figure 4.4	Time course changes in levels of (A) COOH-celecoxib (M2 metabolite) in 6 different horses and (B) the mean (\pm SEM) levels of celecoxib (solid squares), OH-methyl celecoxib (solid triangles) and COOH-celecoxib (asterix) in urine samples.	112
Figure 4.5	Time course changes of faecal excretion of celecoxib (solid squares) and its M2 metabolite COOH-celecoxib.	114
Figure 4.6	Original chart recordings of (A) Collision induced dissociation chromatogram of celecoxib and (B) the corresponding mass spectrum of authentic standard of celecoxib acquired in the negative ion mode.	115
Figure 4.7	Original chart recordings of (A) collision-induced dissociation chromatograms of celecoxib and (B) the corresponding mass spectrum of authentic standards of celecoxib acquired in the positive ion mode.	116
Figure 4.8	Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of carboxylic acid metabolite acquired in negative ion mode of celecoxib obtained from urine samples 24 hours post administration.	118
Figure 4.9	Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of hydroxylmethyl metabolite acquired in negative ion mode of celecoxib obtained from urine samples after 24 hours post administration.	119
Figure 4.10	Original chart recordings showing (A) chromatogram and (B) mass spectrum of hydroxyl celecoxib and carboxyl celecoxib metabolites of celecoxib acquired in positive ion mode from in urine samples after 24 hours administration.	120
Figure 4.11	Original chart recordings of chromatograms showing (A) the retention time of zaleplon (IM), (B) the absence of any signal at the RT of etoricoxib, (C) the peak for zaleplon (IS) and (D) the	122

	peak for etoricoxib spiked in blank plasma.	
Figure 4.12	Time course plasma excretion curves of etoricoxib given as an oral administration at a dose of either 0.5, 1 or 2 mg/kg b wt.	126
Figure 4.13	Time course changes in the levels of etoricoxib (solid triangles) and its metabolite, OH-methyl etoricoxib (solid squares) in plasma for horses.	128
Figure 4.14	Time course changes in levels of etoricoxib (solid diamond), OH-methyl etoricoxib (solid squares) and COOH-etoricoxib (solid triangles) and etoricoxib – 1-N-oxide (solid circles) in urine samples.	129
Figure 4.15	Time course of hydrolyzed urine excretion of etoricoxib and its metabolites.	131
Figure 4.16	Time course of hydrolyzed faecal excretion of etoricoxib and its metabolites.	132
Figure 4.17	Original chart recordings showing (A) collision- induced dissociation chromatograms of etoricoxib and (B) the corresponding mass spectrum of authentic standard of etoricoxib acquired in the positive ion mode.	134
Figure 4.18	Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of 6'-hydroxymethyl metabolite acquired in positive ion mode of etoricoxib obtained from hydrolyzed urine samples 24 hours post administration	135
Figure 4.19	Original chart recording showing (A) extracted ion chromatograms and (B) mass spectrum of glucuronic acid metabolite of 6'-hydroxymethyl etoricoxib acquired in positive ion mode and obtained from urine samples 24 hours post administration.	136
Figure 4.20	Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of 6'-carboxylic acid metabolite of etoricoxib obtained from urine samples from horses.	138
Figure 4.21	Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of hydroxymethyl-1'-N-oxide metabolite of etoricoxib in urine samples from horses.	139

CHAPTER –V: Metabolic profiling of celecoxib and etoricoxib using liver microsomes and tissues

Figure 5.1	Original chart recordings showing the LC/MS/MS chromatogram (above) and SIM fragment ion (below) of (A) etoricoxib (m/z 280), (B) 6'-hydroxymethyl etoricoxib (m/z 278), (C) 6'- carboxy etoricoxib (m/z 328) and (D) 6'-hydroxymethyl-etoricoxib-1-N-oxide (m/z 330) obtained after	153
------------	--	-----

etoricoxib incubation with horse liver microsomes.

- | | | |
|------------|---|-----|
| Figure 5.2 | Original chart recording showing the chromatograms and SRM fragment ion of (A) celecoxib, (B) the absence of hydroxy-celecoxib shown as no characteristic peak and (C) the absence of carboxy celecoxib shown as no characteristic peaks in horse liver microsomal incubation | 154 |
| Figure 5.3 | Original chart recordings of chromatograms and corresponding SIM fragment ions of (A) etoricoxib, (B) 6'-hydroxymethyl etoricoxib, (C) 6'-carboxy etoricoxib and (D) 6'-hydroxymethyl-etoricoxib-1'-N-oxide identified from incubation of etoricoxib with camel liver tissues | 157 |
| Figure 5.4 | Original chart recordings of camel liver tissue <i>in vitro</i> metabolism of celecoxib showing (A) chromatographic peak for celecoxib, (B) chromatographic peak for hydroxy celecoxib and (C) characteristic chromatographic peak for carboxy celecoxib | 158 |

List of Tables

Chapter I General Introduction

Table 1.1	Different classes of NSAIDs with examples	5
Table 1.2	Prostaglandins and their physiological roles in major organ systems and tissues in the body.	8
Table 1.3	Properties of COX-1 and COX-2	9
Table 1.4	Reaction classes of Phase I and Phase II metabolism	46

Chapter II Materials and method

Table 2.1	Details of the <i>in vivo</i> study design	65
-----------	--	----

Chapter – IV Pharmacokinetics and metabolism of celecoxib and etoricoxib in horses

Table 4.1	Intra and inter-day variability of the LC/MS assay for determination of celecoxib in three different concentrations in plasma	106
Table 4.2	Calculated pharmacokinetic parameters of celecoxib following oral administration of 2 mg/kg in horses	107
Table 4.3	Within and inter-day variability of the LC/MS assay for determination of etoricoxib in three different concentrations in plasma	121
Table 4.5	Concentration of either celecoxib or etoricoxib and their metabolites in blood, urine and faeces	146

Chapter –V Metabolic profiling of celecoxib and etoricoxib using liver microsomes and tissues

Table 5.1	Comparison of metabolites formed <i>in vivo</i> and <i>in vitro</i>	139
-----------	---	-----

Abbreviations

µg/ml/hour	microgram/milliliter/hour
µM	micromole
AAS	atomic absorption spectrometry
ADME	absorption distribution metabolism and elimination
ALB	albumin
ALT	alanine aminotransferase
APCI	atmospheric pressure chemical ionization
APCI/MS/MS	atmospheric pressure chemical ionization/ mass spectrometry
APPROVE	adenomatous Polyp Prevention on Vioxx
ARCI	Association of Racing Commissioners International
AST	aspartate aminotransferase
AUC _{total}	area under the curve total
BASO	basophils
BUN	Blood urea nitrogen
BUTE	phenylbutazone
bwt	body weight
Ca ²⁺	calcium
CID	collision induced dissociation
CK	creatinekinase
Cl	clearance
Cl ⁻	chloride
CLASS	Celecoxib Long term Arthritis Safety Study
C _{max}	maximum concentration
CNS	central nervous stimulant
CO ₂	carbon dioxide
CONDOR	Celecoxib versus omeperazole and diclofenac in patients with osteoarthritis and rheumatoid arthritis
COX	cyclooxygenase

COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
COX-3	Cyclo-oxygenase -3
CREA	creatinine
Cu ²⁺	copper
CYP	cytochrome
Da	dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDGE	Etoricoxib Versus Diclofenac Sodium Gastrointestinal Tolerability and Effectiveness
EOS	eosinophils
EPO	Erythropoietin
ER	endoplasmic reticulum
EU	European Union
FDA	Federal Drug Administration
Fe ²⁺	iron
GC	gas chromatography
GGT	gamma-glutamyl transpeptidase
GI	gastrointestinal tract
Hb	haemoglobin
HCX	highly cation exchange
HF	hydrogen fluoride
HPLC	high performance liquid chromatography
HPLC/APCI-MS	high performance liquid chromatography/Atmospheric pressure chemical ionization – mass spectrometry
hr	hour
IC ₅₀	half maximal inhibitory concentration
IM	intramuscular

IT	ion trap
IV	intravenous
K ⁺	potassium
kDa	kilo dalton
kg/bwt	kilogram/body weight
KH ₂ PO ₄	potassium dihydrogen phosphate
LC	liquid chromatography
LC/MS/MS	liquid chromatography/mass spectrometry
LLOQ	lower limit of quantitation
LOX	lipooxygenase
LYM	lymphocyte
M	molar
m/z	mass divided by charge
M1	metabolite 1
M2	metabolite 2
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MEDAL	Multinational Etoricoxib and Diclofenac Arthritis Long-term
mg/kg/bwt	milligram/kilogram/bodyweight
Mg ⁺	magnesium
ml	milliliter
mM	millimolar
MONO	monocytes
mRNA	messenger ribonucleic acid
MS	mass spectrometry
Na ⁺	sodium

NADPH	nicotinamide adenine dinucleotide phosphate
NEU	neutrophil
ng/ml	nanogram/millilitre
NIST	National Institute of Standards and Technology
NMDA	N-Methyl D-Aspartate
NO	nitric oxide
NSAID	non steroidal anti inflammatory drug
OA	osteoarthritis
OFN	oxygen free nitrogen
P450	Cytochrome P450
PCV	packed cell volume
PG	prostaglandin
PG. G/H synthase	Prostaglandin G/H synthase
PGE ₂	Prostaglandin E ₂
PGF ₂	Prostaglandin F ₂
PGH ₂	Prostaglandin H ₂
PGI ₂	Prostaglandin I ₂
pH	power of hydrogen
PHOS	phosphate
PK	Pharmacokinetics
pKa	acid dissociation constant
PLT	platelet
Q	quadrupole
QqQ	triple stage quadrupole
Q-TOF	quadrupole time of flight
RA	rheumatoid arthritis
RBC	red blood cells

RF	radio frequency
RT	retention time
SAA	serum amyloid A
SEM	standard error of mean
SIM	selected ion monitoring
SO ₂	sulphur dioxide
SRM	selected reaction monitoring
SUCCESS	Successive Celecoxib Efficacy and Safety Study
t _(1/2)	terminal half life
TARGET	Therapeutic Arthritis Research and Gastrointestinal Event Trial
TLC	thin layer chromatography
T _{max}	time for maximum concentration
TNF- α	tumour necrosis factor alpha
TOF	time of flight
TP	total blood plasma protein
TXA ₂	thromboxane A ₂
UAE	United Arab Emirates
UV-Vis	ultraviolet-visual
V _d	volume of distribution
VIGOR	Vioxx Gastrointestinal Outcome Research
w/v	weight/volume
WBC	white blood cells
WILEY	John Wiley & Sons, Inc online mass spectral library
Zn ²⁺	Zinc

CHAPTER -1

GENERAL INTRODUCTION

1.1. Historical Perspective of Horse Racing:

Horse racing is one of mankind's most ancient sports and it is often referred to as the sport of Kings. It was the prehistoric nomadic tribes of Central Asia who domesticated the horse around 4500 BC. Horse racing has been a sport in the UK and the rest of the world and this can be traced to the 12th century, when the English Knights brought back swift Arabian horses on their return from their crusades (Dowie, 1977).

Horse racing became a professional sport during the reign of Queen Anne (1702-1714). England became the centre of horse racing, offering large purses, and thereby attracting the best racehorses (Gardner, 2006). These purses made breeding and training of horses more profitable. The sport expanded so rapidly, that it needed a Central Governing Authority, to regulate race meetings, racecourses and horse breeding. The Governing body was set-up in 1750, and it was called the Jockey Club, which exercised complete control over English racing. Modern day racing has flourished because of legalized gambling. This has, however, brought in many crooked ways of winning the purse by altering the performances of horses (Berschneider & Richter, 1980).

Horses are supposed to win a race because of their natural speed and endurance, but “doping” – (employment of an illegal substance to enhance the horse's natural capabilities) has become the shortcut to increase the chances of a horse to win the race or purse. The most commonly abused substances in horse racing are the anabolic steroids, narcotic analgesics like opium and morphine, erythropoietin (EPO), caffeine, butazolidin and the common baking soda (sodium bicarbonate). All these and hundreds of other substances (including substances not on the prohibited list of the Association of Racing Commissioners International) may not be used on horses that are racing (ARCI list of prohibited substances 2009).

Routine dope testing in Britain started in 1963, with the introduction of paper and thin layer chromatography (TLC) and later in 1970s by gas chromatography (GC) and ultra violet spectrophotometry (UV-Vis) (Clarke & Moss, 1976). With the advancement of technology, mass spectrometers have become available which helps the racing chemist in identifying trace amounts of prohibited substances present in blood, urine, faeces and hair.

1.2. Inflammation and drug treatment

Racing of horses involves constant rigorous exercise and training of the animals leading to bone and joint pain and fatigue. The demands of athletic activity take their toll on the body of the horse, particularly the joints, tendons, bones and muscles leading to the release of endogenous intercellular mediators and these in turn cause inflammation (Paulekas & Haussler, 2009; Jenkins, 1987; Driessen *et al.*, 2010).

Pain is a common and distressing phenomenon (Merskey, 1986). The International Association for the study of pain has defined pain as “an unpleasant sensory and emotional experience associated with either actual or potential tissue damage, or described in terms of such damage” (Bonica JJ, 1979). Pain, if not treated, will lead to psychological distress in both humans and animals (Cambridge *et al.*, 2000). It is often thought that arthritis is a human affliction, but it is very common in horses, which ends its racing career leading ultimately to lameness and subsequently, to the death of the animal.

1.3. Treatment of pain

The assessment and alleviation of pain in animals is an important role of the Veterinary Surgeon in the field of Veterinary Medicine (E.J.Love, 2009). Large animals like horses cannot communicate verbally, thus in Veterinary Medicine, the Veterinarian often relies

on physiological indices and behavioural and locomotor changes of the animals (Flecknell, 2008). The behavior of a horse and interactions can be unique to the type of pain the animal is experiencing. A horse's reaction to pain is dependent upon its personality and the degree of pain. The most popular medications used in such situations are the nonsteroidal anti-inflammatory drugs also known by the acronym NSAIDs. Various types of analgesics such as local anaesthetics, opioids, N-Methyl D-Aspartate (NMDA) receptor antagonists, non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin, ibuprofen etc., have been found to be effective in controlling pain, of which opioids and NSAIDs are the main analgesics in animal practice (Slingsby *et al.*, 2006; Vane & Botting, 2000).

The well-known class of NSAID medications is aspirin, or acetylsalicylic acid, first synthesized in 1899. More than 3,000 years ago extracts of myrtle plant was used to treat pain and inflammation, which was later found to contain salicylic acid (Hammerschmidt, 1998). Hippocrates, the father of modern medicine, used the bark of willow, which also contains salicylic acid to relieve joint pain (Limmroth *et al.*, 1999). Felix Hoffman (1868–1946), a scientist employed by the Pharmaceutical Company Bayer, modified the salicylic acid extracted from plant sources to produce aspirin, which became the largest selling pharmaceutical product in history (Vonkeman, 2008).

1.4. Different Chemical Groups of NSAIDs

NSAIDs are divided into six different classes based on their chemical structures. Though they are different in structure, they nevertheless, share a common therapeutic profile and mechanism of action (Vane & Botting, 2003). The major classes are shown in table 1.1. Of these classes of drugs, aspirin belong to the salicylic group constituting to the oldest NSAID that has been in the market for over 100 years. The most recent ones are specific inhibitors of COX-2.

Table 1.1: Different classes of NSAIDs with examples .

Classes	Examples
Acetyl salicylic acid	Aspirin
Acetic acid	Diclofenac, indomethacin, ketorolac, sulindac and tolmetin
Fenamates	Meclofenamate and mefenamic acid
Oxicam	Piroxicam, meloxicam, tenoxicam and lornoxicam
Propionic acid	Ibuprofen, Ketoprofen and naproxen
Coxib	Celecoxib, rofecoxib, valdecoxib, parecoxib and etoricoxib

1.5. Mechanism of Action of NSAIDs

Non-steroidal anti-inflammatory drugs have been used in various forms and for various ailments for more than 3,500 years without knowing the mechanism of action of these drugs (Vane, 2000). Later, it was revealed that all these chemically varied drugs reduce the formation of prostaglandins (see Figure.1.1). The anti-inflammatory activity of NSAIDs is based on the inhibition of prostaglandin synthesis. NSAIDs directly act on prostaglandin synthesis to inhibit or reduce its synthesis and subsequent production (Moncada *et al.*, 1975).

Prostaglandins are powerful signaling lipid (Warden, 2005) agents in the human body (Van der Bijl & Van der Bul, 2003). The two-dozen or so members of this family of small lipid messengers carry out many important physiological functions including vasodilation, vasoconstriction, acid secretion, broncho-constriction, inflammation, uterine contractions, pain perception, fever and several other functions (Kiefer & Dannhardt, 2004). Prostaglandins are involved in bringing about and maintaining the

inflammatory processes by increasing vascular permeability and amplifying the effects of other inflammatory mediators such as kinins, serotonin and histamine (Vane & Botting, 1998b). Therefore, the increased blood flow generates the heat and redness of inflammation. Hence, controlling the formation of some prostaglandins can reduce the swelling, heat and the pain of inflammation (Claria, 2003). However, not all prostaglandins are harmful for the human body (Gnllner *et al.*, 1980). Some of them are important in protecting the stomach lining, promoting the clotting of blood, regulating salt and fluid balance and maintaining blood flow to the kidneys. Table 1.2 shows the different classes of prostaglandins (PGs) and their physiological roles in the different organs and tissues of the body.

Goldblatt and Von Euler in the early 1930s independently discovered a fatty acid in human seminal vesicle having potent vasoactive properties in rabbit and guinea pigs (Sun *et al.*, 1977). This compound was named prostaglandin (PG) because it originated from the prostate gland (Voneuler, 1983). In 1964 the structures of prostaglandins E (PGE) and prostaglandin F (PGF) were proposed and it was demonstrated that they were produced from the essential fatty acid, arachidonic acid (Bergstroem *et al.*, 1964). Around 1972, Piper and Vane demonstrated that NSAIDs prevented prostaglandin production and the mechanism of action of NSAIDs was proposed. It was then demonstrated that NSAIDs work by inhibiting the cyclooxygenase enzymes (COX) or prostaglandin G/H synthase (Ferreira *et al.*, 1973; Vigdahl & Tukey, 1977). This study by Piper and Vane in 1972 led to the role of aspirin in inducing hyperacid secretion in the stomach and subsequent ulcer formation in the stomach. In 1982 Sir John Vane was awarded the Nobel prize, sharing the recognition with Sune K. Bergström and Bengt I. Samuelsson, for showing that aspirin works by blocking prostaglandin synthesis and for discovering prostacyclin and its biological significance (Harding, 2004).

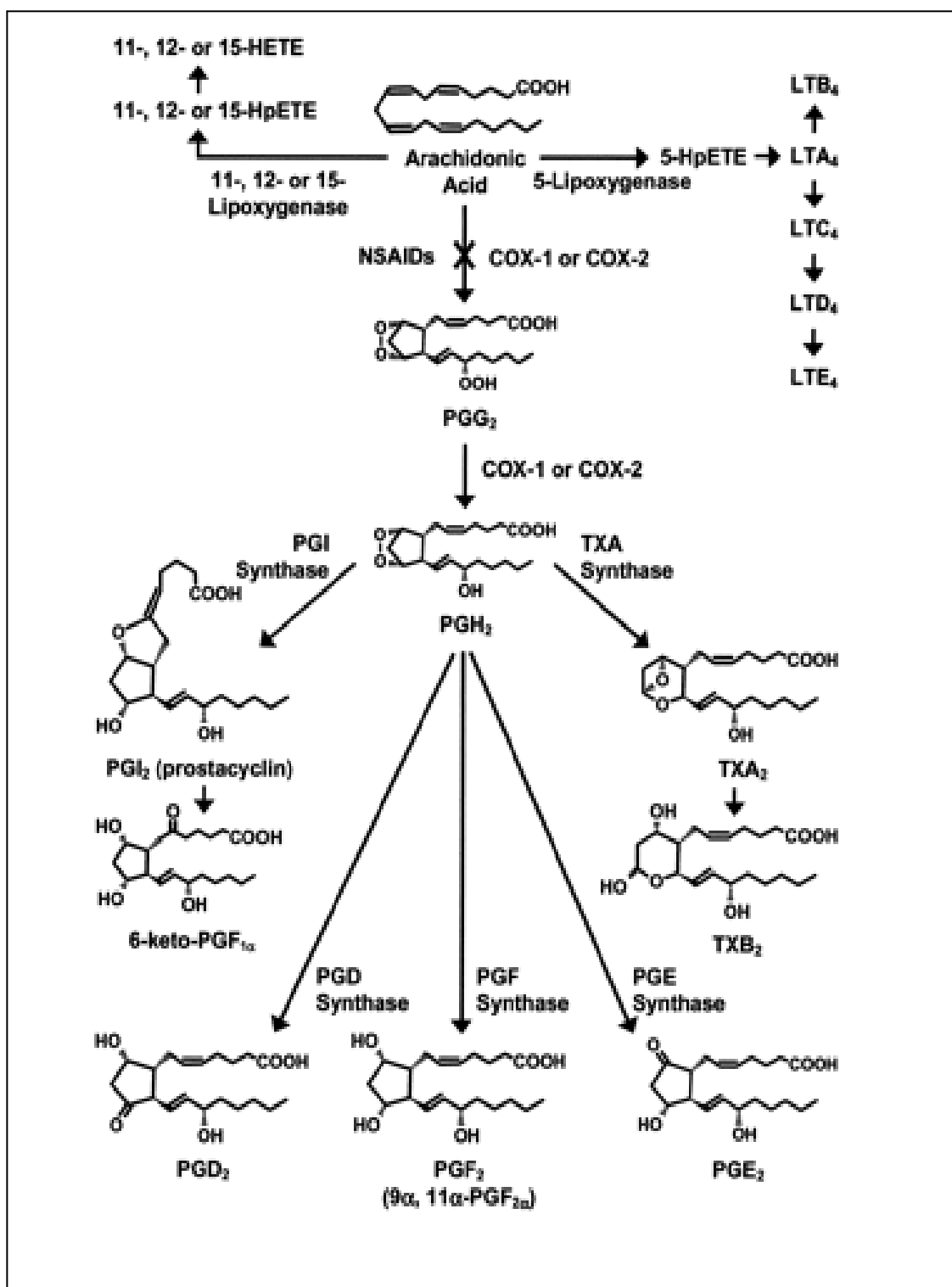


Figure 1.1: The arachidonic acid cascade. The fate of arachidonic acid in cells as it is metabolized by lipoxygenases to HETEs or by cyclooxygenases to form prostaglandin H₂ via the short-lived hydroperoxyl-containing intermediate prostaglandin G₂. (Taken from Vane et al, 2002).

The process of COX pathway begins with arachidonic acid, an unsaturated 20-carbon fatty acid, found in the cell membranes as a phospholipid ester (see Figure. 1.1). The metabolism of arachidonic acid diverges down two main pathways, the cyclooxygenase (COX) and the lipoxygenase (LOX) pathways (Claria, 2003). Both pathways are important to the inflammatory process. Lipoxygenases, like cyclooxygenases, are enzymes that catalyze the stereo specific insertion of molecular oxygen into various positions in arachidonic acid and acts as the key enzyme in the leukotriene pathway (Fiorucci *et al.*, 2003). However, the action of cyclooxygenase pathway is of principal interest in understanding the action of NSAIDs.

NSAIDs exert their therapeutic action by inhibition of cyclooxygenase or prostaglandin endoperoxidase synthase (PGs). The key regulatory step is the conversion of arachidonic acid to PGG₂, which is then reduced to an unstable endoperoxidase intermediate, PGH₂ (Samuelsson *et al.*, 2007). Specific PG synthases metabolize PGH₂ to at least five structurally-related bioactive lipid molecules which include thromboxane A₂ (TxA₂) in platelets (platelet activation and vasoconstriction), PGE₂, PGD₂ and prostacyclin (PGI₂) in the kidney (salt and water excretion), PGE₂ in joints (inflammation and pain), PGI₂ in endothelial cells (platelet inhibition and vasodilatation) and PGE₂ in the central nervous system (pain and fever) (Abramovitz & Metters, 1998; Smith, 2000; FitzGerald & Patrono, 2001).

1.6.Cyclooxygenase isoform, structure and function

Figure 1.1 shows the arachidonic acid cascade pathway and table 1.3 shows the properties of COX-1 and COX-2. The pathway involves the synthesis of the different prostaglandins from arachidonic acid and employing a number of enzymes including PG synthase. Though the involvement of the COX pathway has been reported since the

Table 1.2: Prostaglandins and their physiological roles in major organ systems and tissues in the body.

Systems	Mediator(s)	Major Site(s) of Synthesis	Primary Effect(s)
Cardiovascular	Prostacyclin	Endothelial cells	Vasodilatation
	Thromboxane	Platelets	Vasoconstriction
Renal	Prostacyclin	Renal cortex	Vasodilatation
	PGE ₂	Renal medulla	Salt and water excretion
Gastrointestinal	PGE ₂	Gastric mucosa	Cytoprotection
Hematologic	Prostacyclin	Endothelial cells	Platelet de-aggregation
	Thromboxane	Platelets	Platelet aggregation
Respiratory	Prostacyclin	Endothelial cells	Vasodilatation
Musculoskeletal	PGE ₂	Osteoblasts	Bone resorption ,bone formation
Reproductive	PGE ₂	Seminal vesicles	Erection, ejaculation, sperm transport
	PGE ₂ , PGF ₂	Fetal membranes Uterus	Parturition/labor, menstruation, fertilization, ovulation
Neurologic	PGE ₂	Unknown	Fever, hyperalgesia

early 70's, (Miyamoto *et al.*, 1976), the detail concept of the COX pathway evolved only in the early 90's (Merlie *et al.*, 1988; Yokoyama *et al.*, 1988) which indicates that the membrane bound enzyme, cyclooxygenase has at least two types of isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). These isoforms are encoded by separate genes and they are likely to have different cellular functions (Sanghi & MacLaughlin 2006). The genes for COX-1 and COX-2 are located on human chromosomes 9 and 1, respectively (Wu & Liou, 2005).

Table 1.3. Properties of COX-1 and COX-2

	COX-1	COX-2
Gene	22 kb, chromosome 9 mRNA 2.8 kb	8 kb, chromosome 1 mRNA 4.3 (unstable)
Expression	Constitutive	Inducible by multiple compounds including growth factors, LPS and mitogens
Location	Platelets Stomach Kidney	Macrophages Leukocytes Fibroblasts, endothelium, brain and ovaries
Function	Housekeeping	Inflammatory

Structurally, COX-1 and COX-2 are haeme containing enzymes with COX-1 having 602 amino acids and COX-2 with 604 amino acids and consisting of a long narrow channel with a hairpin bend at the end. Each class of cyclooxygenase lends itself to produce different types of prostaglandins (Meade *et al.*, 1993).

Significant differences exist in the subcellular location of COX-1 and COX-2. COX-1 is equally distributed in the endoplasmic reticulum (ER) and nuclear envelope (NE), while COX-2 is twice as concentrated in nuclear envelope as in the endoplasmic reticulum (Morita *et al.*, 1995). The structural difference between COX-1 and COX-2 has been

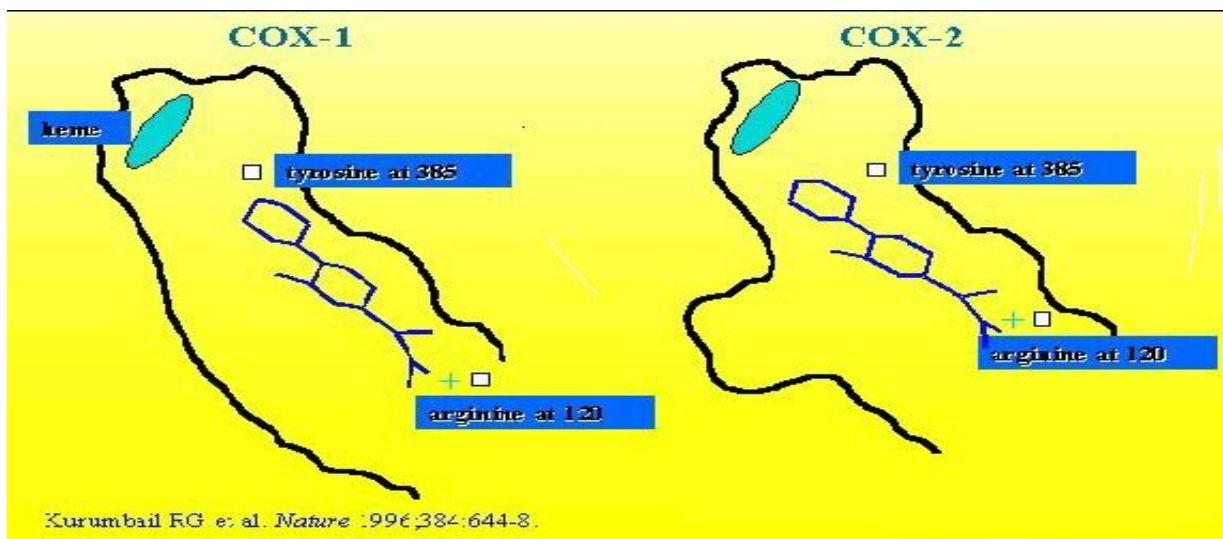


Figure 1.2: Structure of COX-1 and COX-2 (Kurumbail *et al.*, 1996).

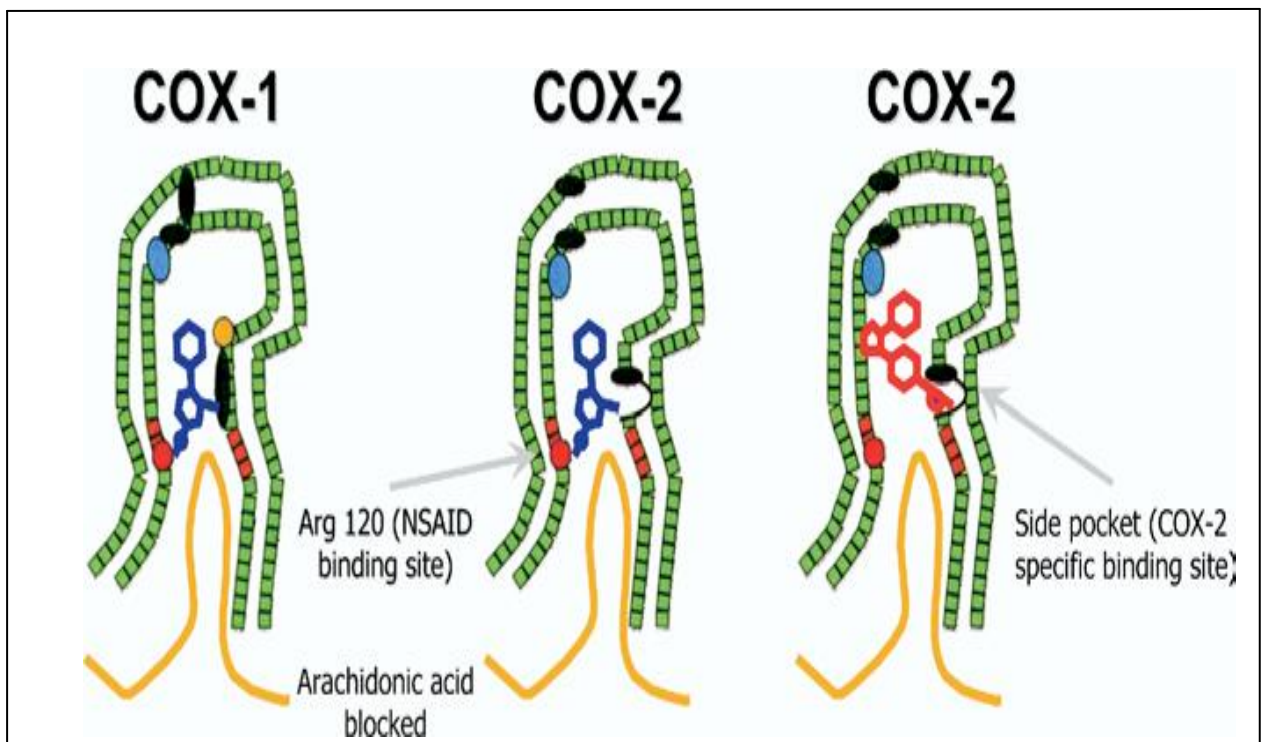


Figure 1.3: Schematic representation of the inhibition of COX-1 by a nonselective NSAID (central black figure). The entrance channel to COX-1 is blocked by the NSAID. Binding and transformation of arachidonic acid within COX-1 is prevented.

Middle: inhibition of COX-2 by a nonselective NSAID (central black figure).

Right: inhibition of COX-2 by COX-2 selective NSAID. The COX-2 side pocket allows specific binding of the COX-2 selective NSAID's rigid side extension. The entrance channel to COX-2 is blocked. The bulkier COX-2-selective NSAID will not fit into the narrower COX-1 entrance channel, allowing uninhibited access of arachidonic acid into COX-1 (Taken from Hawkey. 1999).

shown schematically in figure 1.2. Figure 1.3 shows the schematic representation of the inhibition of COX-1 by nonselective NSAID and selective inhibition of COX-2 by COX-2 selective NSAIDs.

Recently, COX-3, derived from the COX-1 gene by splicing the COX-1 mRNA has been found to be sensitive to acetaminophen (Simmons *et al.*, 2005). Acetaminophen, unlike other NSAIDs, is known to exhibit weak inhibition of both COX-1 and COX-2 enzymes at therapeutic concentrations. They have been found to inhibit COX-3, the brain isoform of COX (Botting & Ayoub, 2005). The cells, in order to synthesize mature mRNA to encode COX-1 protein, remove a particular stretch of sequence, intron 1, and this mRNA intron 1 is included in COX-3. COX-1 has 10 introns, including intron 1, COX-2 has 9 and COX-3 has the additional intron 1 of COX-1 as an exon. COX-3 is about 65 kDa in molecular weight (Chandrasekharan *et al.*, 2002).

1.7. Cyclooxygenase-1: the constitutive isoform

The isoform COX-1 is expressed constitutively in many tissues. This in turn carries out some homeostatic functions in the body by the action of physiological stimuli, thereby converting arachidonic acid into prostaglandins with specific 'housekeeping' functions (Seibert *et al.*, 1997). These prostaglandins in turn stimulate normal physiological functions including stomach mucus production, kidney water excretion, platelet formation, macrophage differentiation and others (Vane *et al.*, 1998). In humans and most other animal species, the cytoprotective prostaglandins in the stomach are synthesized by COX-1. This cytoprotective action is due to vasodilatation, to enhance mucosal blood flow (Kargman *et al.*, 1996).

In a study Miyamoto *et al.*, (1976) used isoelectric focusing to identify and purify cyclooxygenase-1 from bovine and sheep vesicular glands. They found it to be a

membrane bound homo-dimer of 70 kDa. This protein, together with free or protein bound haeme, contained both the cyclooxygenase and peroxidase activity to form, PGG₂ and PGH₂. The primary structure of cyclooxygenase-1 was later determined from the complementary DNA sequence of 2.7 kilo bases (William L.Smith, 2000). Inhibition of COX-1 would therefore interrupt some normal physiological functions (Shi & Klotz, 2008).

1.8. Cyclooxygenase-2: the inducible form

In contrast to COX-1, the COX-2 enzyme is mainly used as an inducible isoform of cyclooxygenase and it is produced primarily at sites of inflammation (Birbara *et al.*, 2003). COX-2 is known to be the dominant isoenzyme in inflamed tissues where it is induced by a number of cytokines, including interleukin1, tumour necrosis factor alpha (TNF- α), bacterial toxins etc, with the apparent exclusive role of producing pro-inflammatory prostaglandins (Scher & Pillinger, 2009). Cells that express COX-2 in response to mitogens are endothelial cells, smooth muscle cells, chondrocytes, fibroblast, monocytes, macrophages and synovial cells (Crofford, 2000). Initially, it was believed that this was due to an increase in supply of arachidonic acid, but in 1990, it was demonstrated that the increase in prostaglandin formation following exposure of isolated cells in culture to inflammatory stimuli (Smith & Marnett, 1991) was due to an increase in cyclooxygenase enzyme expression (Fu *et al.*, 1990). Inhibition of this isoform leads to the control of pain and inflammation (Noble *et al.*, 2000). It is particularly interesting and awakening that the identification of cyclooxygenase-2 was, in many respects, a triumph of molecular biological studies (Seibert & Masferrer, 1994).

The regulation of prostaglandin biosynthesis by COX-1 and COX-2 (known as the general COX concept) is shown schematically in figure 1.4. The figure shows how the different steps in the synthesis of the different PGs and thromboxane from arachidonic

acid employing a number of enzyme precursors including PGG₂ and PGH₂. To combat pain and inflammation, the COX-2 inhibition by NSAIDs is desirable and not the COX-1. Non-specific inhibition of COX-1 and COX-2 can lead to harmful side effects of the NSAIDs (Willoughby *et al.*, 2000).

There may be five different ways in which NSAIDs can inhibit COXs (Hawkey, 1999). These include

- i) Reversible competitive inhibition of both COX-1 and COX-2
(e.g. mefenamic acid, ibuprofen)
- ii) Irreversible acetylation of both COX 1 and COX 2 (e.g. aspirin)
- iii) Slow the time dependent inhibition of both COX-1 and COX-2.
- iv) Some NSAIDs like indomethacin, ibuprofen can cause conformational change at the binding site of COX.
- v) Specific inhibition of COX-2 (celecoxib, rofecoxib etc.).

However, NSAIDs vary in their abilities to inhibit COX-1 and COX-2 at different concentrations and in different tissues (Hwang *et al.*, 2004). Traditional NSAIDs including high-dose aspirin are nonselective and can block both isoforms of COX. Their therapeutic efficacy depends on the degree of inhibition of the COX-2 mediated formation of PGE₂, which causes inflammation of joints, fever and pain in the central nervous system (Laneuville *et al.*, 1994). However, the inhibition of COX-1 mediated PGE₂ formation in the gastric mucosa increases the risk of mucosal damage and gastrointestinal bleeding (John A Cairns, 2007). Figure 1.5. summarizes the structural evolution (precursors, intermediate products and the final NSAID) of some of the major classes of NSAIDs used to treat inflammation and pain in humans. The data shown in figure 1.5 also show the differences in the chemical structures of the NSAIDs.

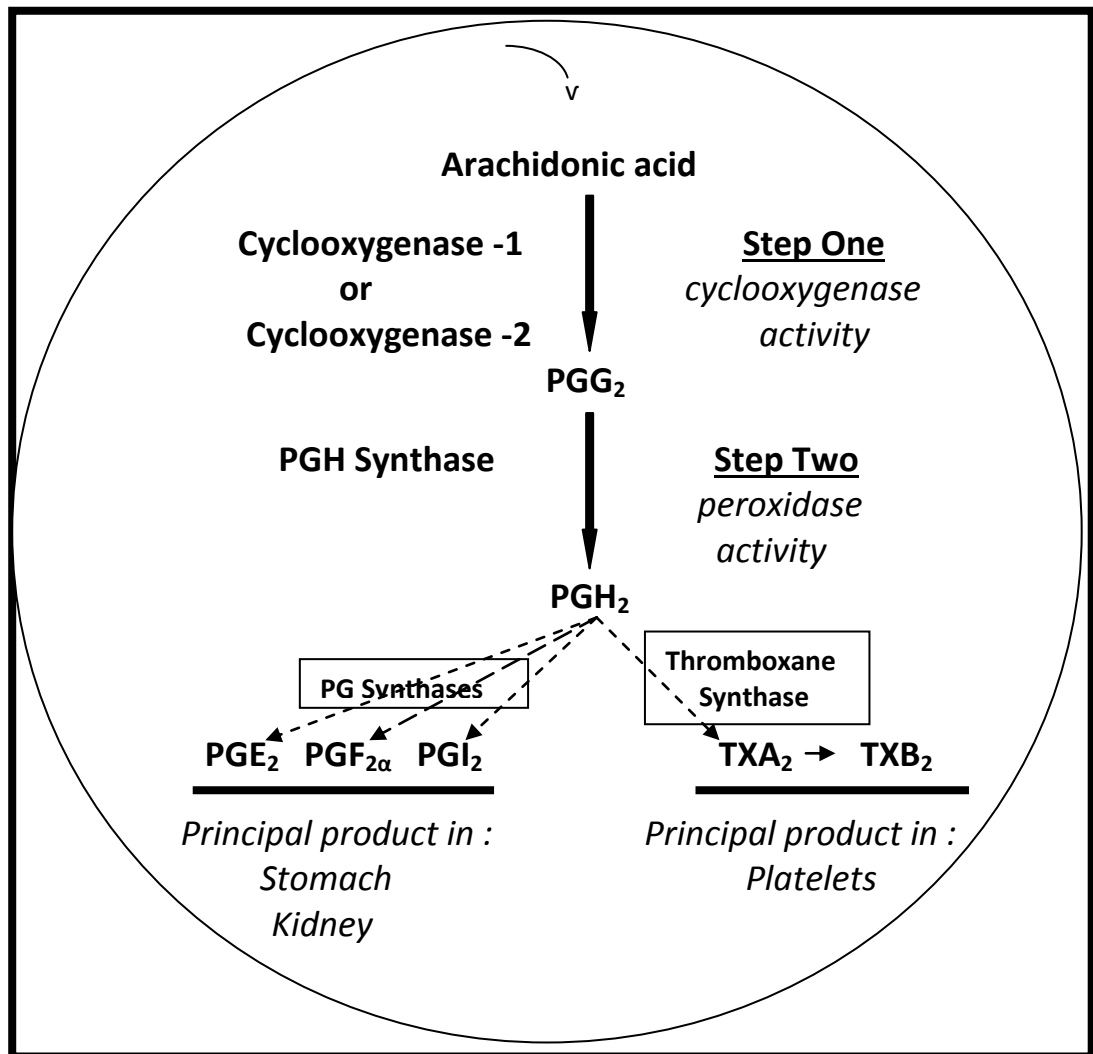


Figure 1.4: The Cyclooxygenase pathway

Much effort has gone into developing nonsteroidal anti-inflammatory drugs (NSAIDs) that selectively inhibit COX-2 rather than COX-1. Thus, the rationale is not to affect the homeostasis functions of the prostanoids preferentially synthesized by COX-1, but in particular to reduce the gastro-intestinal bleeding caused by COX-1 inhibition (Raskin, 1999).

1.9. Some common NSAIDs used to treat horses

The treatment and management of animals with pain, including osteoarthritis in horses and dogs involve both therapeutic and non-therapeutic measures (Otto & Short, 1998). A key consideration of non-therapeutic measure is the control of body weight in dogs. Present day Veterinary Clinicians recommend mild to moderate exercise to maintain joint motility in horses (Paulekas & Haussler, 2009). However, therapeutic intervention is also necessary (Lees *et al.*, 1999). Figure 1.6 shows some commonly used NSAIDs in horses. The figure shows the chemical structure of each compound for comparison.

NSAIDs are commonly used in Veterinary Medicine for the treatment of inflammation of musculoskeletal and other tissues (e.g. spondylitis, laminitis, mastitis), endotoxic shock and colic in the horse and for the control of pain, associated with either trauma or surgery (Stewart *et al.*, 2008). These drugs may favourably influence the course and outcome of certain diseases and disorders. The ability of non-steroidal anti-inflammatory drugs to suppress inflammation and subsequent tissue damage is important, since the inflammatory process may result in organ damage that renders the animal either unprofitable or useless for production (Kopcha & Ahl, 1989).

Also, the suppression of pain, which causes distress to the animal is an important pharmacological property of NSAIDs (Driessen 2007). The chemical structures of some

commonly used NSAIDs are given in Figure 1.5. From the figure, it can be seen that aspirin had formed the prototype for the development of different analgesic and antipyretic drugs.

Medications for the control of equine pain can be selected from NSAIDs, such as corticosteroids, opioids, α_2 -adrenergic agonists, anaesthetics and other CNS response altering agents (Short, 1995). Most NSAIDs have valuable therapeutic properties, and some have a great potential for toxicity. The toxic effects of NSAIDs, in particular, gastric ulceration, is well known in many species of animals (Martineau *et al.*, 2009). Interestingly, equine toxicity seems relatively uncommon, provided the drug is administered at the recommended clinical dose (Lees & Higgins, 1985).

In equine Veterinary Practice, pain management involves the administration of pain inhibitors to the horses (Alexander Valverde, 2005). Commonly used NSAIDs in horses are shown in figure 1.6. Multiple drug classes that can have an impact in transmission of pain in various points of the pain pathway can be used either solely or as a combination (Brunson & Majors, 1987). These medications block the enzymes released by the body's pathway for inflammation, thus reducing inflammation, redness and keeping the activity of the injured cells to a minimum (see Figure.1.4 on PGs synthesis).

Development of a drug specific for a particular animal is tedious, expensive and time consuming and as a result, the drug developed for human use are being used in cattle camel, horses and many small animals (Grave *et al.*, 1992). Most drug preparations intended for use in human have been reported as illicit substances in body fluids of horses at racecourses and at jumping events (Delbeke, 1995).

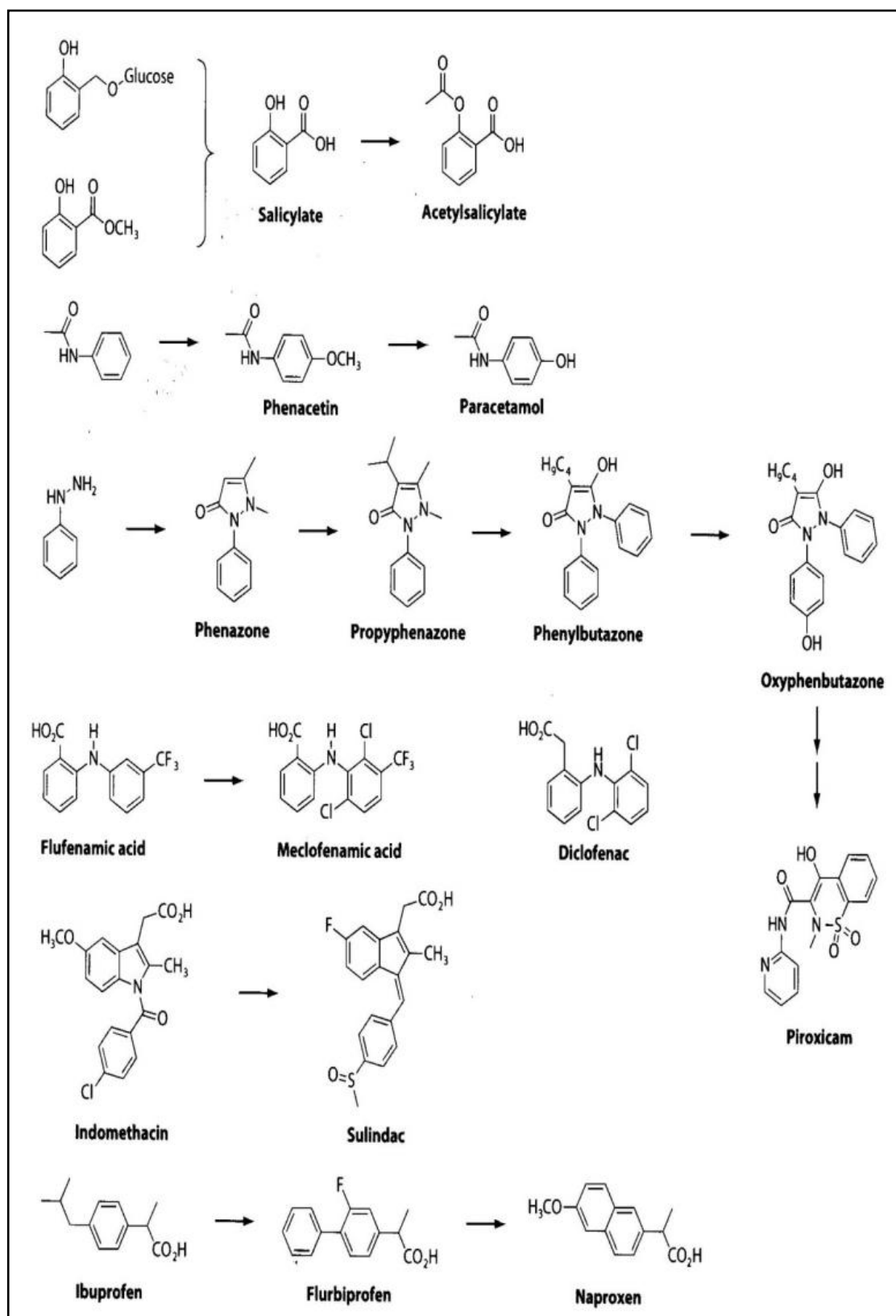


Fig.1.5: Chemical structures and evolution of major classes of NSAIDs prior to discovery of COX-2 (Taken from Marnett, 2009).

Previous studies have shown that 43% of all prescriptions in Veterinary Medicine are human drug preparations and about 19% of those prescriptions are disposed for horses (Delbeke *et al.*, 1994). Drugs like flunixin meglumine, meclofenamic acid, naproxen, and phenylbutazone are approved for horses and are frequently abused in the horse racing industry (Kopcha & Ahl, 1989). This introduction will now review the literature on the use of these different NSAIDs in horses and other animals.

1.9.1. Phenylbutazone

Of the medications for control of equine pain, the NSAIDs have fulfilled an important role in improving the comfort and well being of horses in training and racing. Phenylbutazone has been the drug of choice for treating musculoskeletal disorders since it was introduced to Veterinary Medicine in the 1950s (Kallings *et al.*, 1999a).

Phenylbutazone is an acidic, lipophilic non steroidal agent used extensively to treat horse, since it has analgesic, anti-inflammatory and antipyretic properties (Watson *et al.*, 2004). The metabolites identified to date are oxyphenbutazone and γ -hydroxyphenylbutazone (Tobin *et al.*, 1986). Phenylbutazone acts by inhibiting the cyclooxygenase enzyme system responsible for the synthesis of prostanoids such as PGE₂. It markedly reduces prostanoid-dependent swelling, oedema and hypersensitivity to pain from inflamed tissue. As a result, its main and principal use in horses is to resolve soft tissue inflammation. It is highly protein bound in the plasma with an elimination half-life of 5.5 hours (Lees *et al.*, 1987). Phenylbutazone is given either orally or intravenously. It effectively relieves pain from inflammation of the musculoskeletal system (Mac Allister *et al.*, 1993). It can alleviate lameness for several days following therapy, and it has been used to disguise lameness for the purpose of competitive racing (Coffman & Garner, 1972).

Phenylbutazone administered above a dose of 4.4 mg/kg twice daily has resulted in anorexia, neutropenia, hypoproteinaemia and eventually death in horses (Snow, 1981; Mackay et al., 1983). In blood, if its concentration is greater than 99%, then it is protein bound. It can also interact with other protein bound drugs like warfarin, phenytoin etc., resulting in toxicity. Gastrointestinal effects are the most important adverse effect of phenylbutazone therapy in horses (Jennifer L.Davis, 2009) .

1.9.2. Flunixin

Flunixin is probably the second most widely used NSAID in horses to treat pain and inflammation. It is commonly used as an anti-inflammatory drug for the management of colic, endotoxaemia and musculoskeletal disorders in the horse. Flunixin has a short half-life of 1.6-2.5 hours compared to phenylbutazone and peak plasma concentrations are reached within 30 minutes following oral administrations (Chay *et al.*, 1982). Flunixin is used at a dose of 1.1 mg/kg and given once daily by oral or IV routes. In a study comparing postoperative analgesia of flunixin, phenylbutazone and carprofen Johnson et al., (1993) reported that flunixin had the longest duration of analgesic effect. Furthermore, Moses & Bertone., (2002) showed that the toxic effects of flunixin were found only when approximately five times the daily recommended dose of 1.1 mg/kg was administered and moreover, frequent intramuscular injections on several occasions could cause local tissue irritation and damage.

The most common adverse effects associated with flunixin are vomiting, diarrhoea, lethargy and lack of appetite. Stomach ulcers and kidney impairment are possible with flunixin (MacAllister *et al.*, 1993). Like other NSAIDs, flunixin is highly protein bound. In a study comparing flunixin with other analgesics, it was found that flunixin had the longest duration of analgesic effect of about 12.8 hours (Houdeshell & Hennessey, 1977).

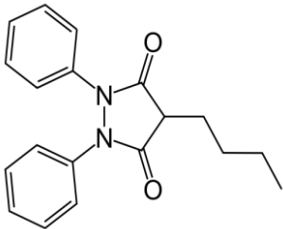
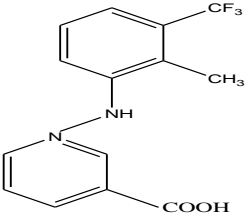
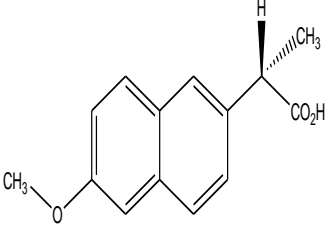
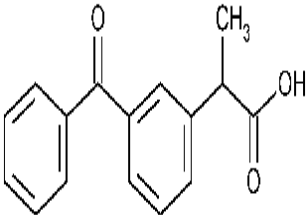
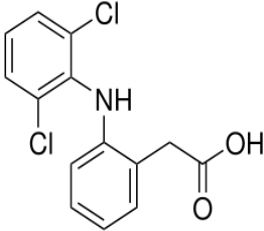
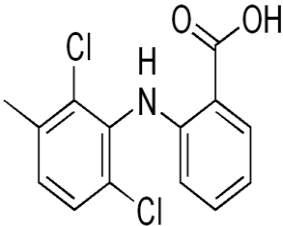
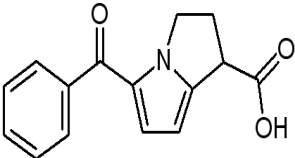
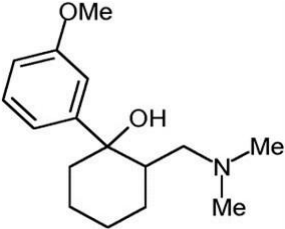
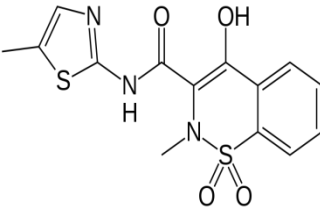
 <p>Phenylbutazone</p>	 <p>Flunixin</p>	 <p>Naproxen</p>
 <p>Ketoprofen</p>	 <p>Diclofenac</p>	 <p>Meclofenamic acid</p>
 <p>Ketorolac</p>	 <p>Tramadol</p>	 <p>Meloxicam</p>

Figure 1.6: Chemical structures of some commonly used NSAIDs in horses to treat pain.

1.9.3. Naproxen

Naproxen is approved by Federal Drug Administration (FDA) in the USA, as an oral granular preparation to be used in horses. Naproxen is administered orally at a dose of 10 mg/kg with a bioavailability in horse is about 50% (Mcdowell & Wickler, 1990). Elimination half-life for the parent drug and its metabolite is approximately 6 hours (Tobin, 1979). Naproxen is well tolerated and 3 times the recommended dose for 6 weeks do not show any toxicity (Lee and Higgins 1985).

1.9.4. Ketorolac

Ketorolac has anti-inflammatory, analgesic and antipyretic properties and it has been used in humans with moderate to severe pain, including post operative and post partum pain. The anti-inflammatory effect of ketorolac appears to be caused mainly by inhibition of prostaglandin synthesis during inflammation (Litvak & Mcevoy, 1990).

In humans, ketorolac is absorbed rapidly and completely after oral administration. The drug is highly protein bound (>99%). The total plasma clearance is approximately 0.03 l/kg and the plasma elimination half-life is 5-6 hours. It is likely that either most or all of the drug-related materials circulating in plasma are in the form of ketorolac (>96%) and the only metabolite found is the pharmacologically inactive p-hydroxyketorolac. In human urine, about 90% of the administered dose is excreted in the form of ketorolac (60%), p-hydroxyketorolac (12%) and glucuronide conjugates (28%) (Mroszczak *et al.*, 1990).

1.9.5. Ketoprofen

Ketoprofen is a propionic acid derivative available both in tablet and injection forms, and it is used widely in the treatment of equine colic and as an analgesic in pain (Longo *et al.*, 1992). Ketoprofen exists as 'R' and 'S' enantiomers having different elimination half lives, but is formulated as a racemic mixture (Jaussaud *et al.*, 1993). The recommended maximum dose is about 2.2 mg/kg body weight per day. It is rapidly absorbed and eliminated with mean serum concentration in horses are 1.58 ng/ml and 1.56 ng/ml following IV and IM administrations and a half life of 2 – 2.5 hours (Brink *et al.*, 1998).

Ketoprofen undergoes hydroxylation to form hydroxyl ketoprofen in horses (Corveleyn *et al.*, 1996). As with other NSAIDs, ketoprofen is metabolized in the liver and eliminated by renal excretion. Erosion or ulcers of the tongue and stomach has been noticed with ketoprofen administration. Some adverse effects of the drug include gastrointestinal tract (GI) up sets which are similar to those induced by other NSAIDs. Other side effects in animals include haepatopathies and renal disease (MacAllister *et al.*, 1993). Due to potential anti-platelet effects, care should be exercised using ketoprofen perioperatively (Adams, 2000)

1.9.6. Carprofen

Carprofen is a chiral non-steroidal anti-inflammatory drug marketed for the relief of pain and inflammation associated with osteoarthritis in horses (Armstrong *et al.*, 1999). Like ketoprofen, carprofen exists as two enantiomers with a $t_{1/2}$ of 18-22 hours. The mechanism of action of carprofen is not fully elucidated (Armstrong & Lees, 1999). However, Benton *et al.*, (1997) demonstrated that carprofen showed moderate inhibition both *in vitro* and *in vivo* studies exhibiting only minimal inhibition (Lees *et*

al., 1994a). Carprofen is usually administered either orally at a dose of 1.4 mg/kg or intravenously at 0.7 mg/kg.

1.9.7. Tramadol

Tramadol is a synthetic, centrally acting analgesic agent with 2 distinct, synergistic mechanisms of action, acting as both a weak opioid agonist and an inhibitor of monoamine neurotransmitter reuptake. It is structurally related to codeine and morphine. Tramadol is used clinically to treat moderate to severe pain in humans (Scott & Perry, 2000). Tramadol is extensively metabolized in humans in the liver, forming O-desmethyl tramadol and N-desmethyl tramadol as metabolites. O-desmethyl tramadol is the major metabolite in humans, while in dogs it is relatively a minor metabolite (Wu *et al.*, 2001).

Intravenous administration of horses with a concentration of 2 mg/kg, showed the elimination half-life to be about 82 minutes, which was shorter than the 5.5 hours, reported in humans. Moreover, the drug is found to be completely absorbed in humans when given orally, while in horses, bioavailability is very low, excluding oral administration (Shilo *et al.*, 2008).

1.9.8. Meloxicam

Meloxicam is an enolic acid NSAID of the oxicam family and is approved for use in animals in Europe, Canada and the United States (Moses & Bertone, 2002). Meloxicam is a long acting, highly potent anti-inflammatory drug used in the treatment of rheumatoid and osteoarthritis (Lees & Higgins, 1985). Meloxicam inhibits COX-2 in preference to COX-1 and show a greater gastrointestinal tolerance when compared to conventional NSAIDs (Prouse *et al.*, 2005).

In human, meloxicam is extensively metabolized with less than 0.25% is excreted as the parent drug and it has a half-life of about 20 hours (Turck *et al.*, 1997). Unlike in humans, meloxicam is not metabolized extensively by Phase 1 biotransformation in the horse. In horses and other animals the main pathway for meloxicam metabolism involves the kidneys where the drug and its two metabolites, hydroxyl meloxicam and carboxy meloxicam are excreted in urine, mainly un-conjugated (Dumasia & Bruce, 2002;Dumasia & Bruce, 2002;Dumasia & Bruce, 2002;Little *et al.*, 2007).

1.9.9. Firocoxib

Firocoxib is the only COX-2 inhibitor approved as a veterinary pharmaceutical drug for the pain management associated with osteoarthritis in horses and dogs. It provides therapeutic efficacy by inhibition of prostaglandin synthesis via selectively binding to the COX-2 isoenzyme (Mccann *et al.*, 2004). Firocoxib is highly selective for COX-2 and has little effect on COX-1 isoenzymes even when administered at higher concentrations (Letendre *et al.*, 2008). It is marketed by MERIAL under the brand name 'EQUIOXX'.

Firocoxib is not a cure for osteoarthritis, but it can help to control pain and inflammation associated with it, and by doing so it can improve the mobility of horses (Doucet *et al.*, 2008). Kvaternick *et al.*, (2007) studied the pharmacokinetics of firocoxib and established that firocoxib is well absorbed after oral administration, with bioavailability of 79%, elimination half life of 30 hours and C_{max} of 75 ng/ml at 3.9 hours. Firocoxib is mainly excreted through urine as an inactive metabolite namely descyclopropylmethylfirocoxib and as a glucuronide conjugate. Recently, Orsini *et al.*, (2012) showed that firocoxib is a safe cyclooxygenase-2 specific NSAID that

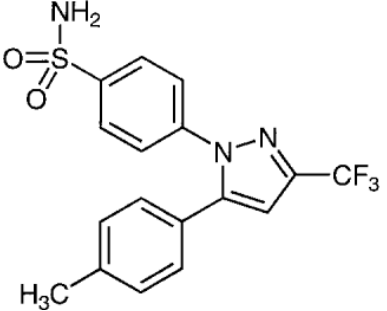
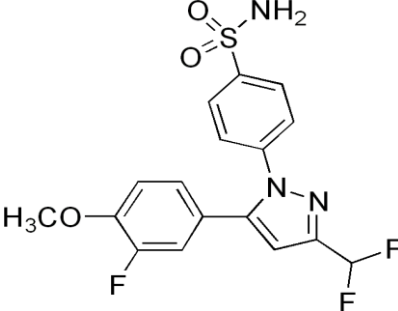
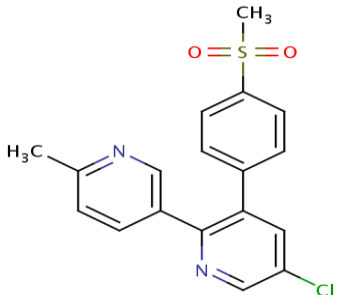
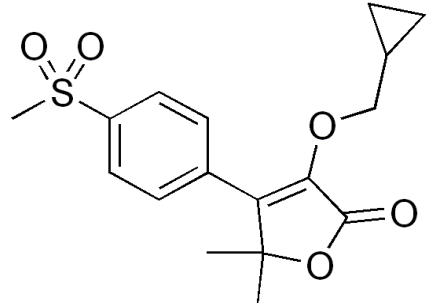
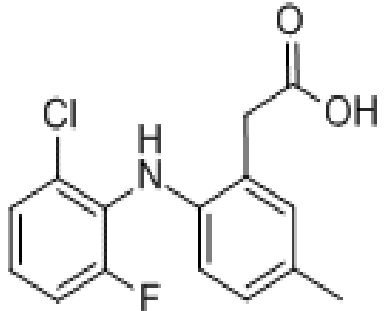
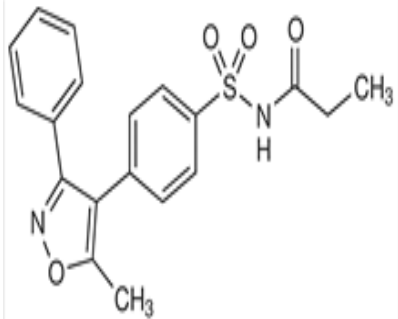
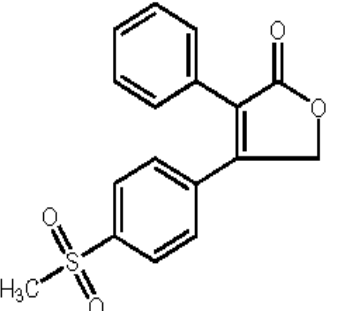
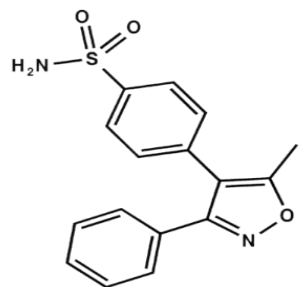
 <p>CELECOXIB</p>	 <p>DERACOXIB</p>	 <p>ETORICOXIB</p>	 <p>FIROCOXIB</p>
 <p>LUMIRACOXIB</p>	 <p>PARECOXIB</p>	 <p>ROFECOXIB</p>	 <p>VALDECOXIB</p>

Figure 1.7: Chemical structure of coxib NSAIDs.

can be used in horses for muscular pain and moreover, horses treated with firocoxib had significant improvement in lameness scores from baseline values.

1.10. New COX-2 Inhibitors

Traditional NSAIDs prescribed to control pain and to treat inflammatory conditions such as osteoarthritis and rheumatoid arthritis produce their anti-inflammatory and analgesic effect by nonselective inhibition of the COX activity leading to pain relief and unwanted side effects. Selective inhibitors of cyclooxygenase-2 (COX-2) have been demonstrated to be clinically effective as anti-inflammatory and analgesic drugs with reduced gastrointestinal toxicity as compared to NSAIDs (Bombardier *et al.*, 2000).

During an inflammatory process, a dramatic increase in COX-2 level normally occurs leading to enhanced production of pro-inflammatory prostaglandins (see Figure.1.4). The expression of COX-2 inhibitors has been extensively studied in small animals, which provided strong evidence that inhibition of COX-2 enzymes can result a decrease in pain conditions (Wilson *et al.*, 2006).

Currently, there is abundant interest in the identification of a COX-2 inhibitor. The aggressive exploration in search of this safer selective COX-2 inhibitor led to the introduction of rofecoxib, celecoxib, valdecoxib, etoricoxib, lumiracoxib and deracoxib. The structures of these COX-2 inhibitors are given in Figure 1.8. Dup697 is the first among these drugs and has a diarylthiophene with a methylsulfone in one aromatic ring (Gans *et al.*, 1990).

Phenylsulfone- and phenylsulfonamide – containing tricyclic molecules have proved to be a fertile area for the development of new COX-2 selective inhibitors (Gauthier *et al.*, 1996). Few among them are the furanone of rofecoxib, the trifluoromethyldiazole of

Celecoxib, the chloropyridine of etoricoxib and the isoxazole ring of valdecoxib (Friesen *et al.*, 1996). Rofecoxib (VIOXX) was withdrawn voluntarily by Merck from the market in September 2004 following an increased cardiovascular risk observed in APPROVE (Adenomatous Polyp Prevention on Vioxx) study (Ruschitzka, 2005). Subsequently, the sale of valdecoxib (Bextra) was also suspended by Pfizer in 2005 due to high incidence of heart attacks and strokes (Nussmeier *et al.*, 2005).

However, CLASS (Celecoxib Long term Arthritis Safety Study) trial conducted on Celecoxib found no increased risk of cardiovascular thrombotic events (Silverstein *et al.*, 2000a). The mechanism underlying the adverse cardiovascular effects associated with the use of COX-2 inhibitors is due to an imbalance between COX-1 derived thrombotic thromboxane A₂ (TXA₂) in platelets and COX-2 derived vaso-protective prostacyclin (PGI₂) in endothelium (FitzGerald, 2004).

Similarly, lumiracoxib did not demonstrate significant cardiovascular side effects in TARGET (Therapeutic Arthritis Research and Gastrointestinal Event Trial), but serious hepatic side effects resulted in withdrawal of lumiracoxib from Australian market followed by the European market (Zarraga & Schwarz, 2007). Currently, celecoxib is the only available coxib commercially available in the US, while celecoxib and etoricoxib are available in the EU countries (Anonymous, 2012).

1.10.1. Pharmacokinetics and Pharmacodynamics of celecoxib and etoricoxib

Cyclooxygenase-2 inhibitors such as celecoxib and etoricoxib are marketed worldwide for the relief of chronic pain in osteoarthritis and rheumatoid arthritis (Antoniou *et al.*, 2007). Figure 1.7 shows the chemical structures of the coxib NSAIDs. Current evidence has shown that that these classes of drugs produce minimal adverse effects in the stomach compared to traditional NSAIDs. Celecoxib was the first COX-2 inhibitor

introduced into clinical practice and is available in different names worldwide since 1999 (Ault, 1998).

Previous studies have investigated the cardiovascular safety of coxibs examining their effects to induce myocardial infarction, stroke and unstable angina. These initial studies have demonstrated that the coxibs administered in high doses and for longer periods of time can induce cardiovascular problem. As such both rofecoxib and valdecoxib were withdrawn from the market (Ruschitzka, 2007). Coxib group of drugs differ in their chemical structure with a complete absence of a carboxylic group which was earlier thought to be a prerequisite for a classic NSAID (Dannhardt & Kiefer, 2001). Thus, celecoxib and valdecoxib possess a sulfonamide group while rofecoxib and etoricoxib have a methylsulfone moiety, which could be responsible for their distinct pharmacokinetic properties (Zarghi & Arfaei, 2011).

1.10.2. Pharmacokinetics and pharmacodynamics of celecoxib

Celecoxib is the first cyclooxygenase-2 inhibitor to be introduced into clinical practice (McCormack, 2011). Celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl]-benzenesulfonamide is a 1,5-diaryl-substituted pyrazole with a pKa of 11.1 and belongs to the first generation of coxibs and it was launched in 1998 (Simon *et al.*, 1998). Currently, celecoxib is approved for the relief of the signs and symptoms of rheumatoid arthritis, osteoarthritis, and for the management of pain (Yoshino *et al.*, 2005). The introduction of celecoxib has been the culmination of the discovery of the COX isoenzymes. This has paved way for the search for molecules that effectively inhibit COX-2 with little or no effect on COX-1. Thus, the major clinical goal to produce NSAIDs that have no effect on the gastrointestinal tract and kidneys was accomplished by the clinical introduction of celecoxib in 1999 (Karim *et al.*, 1997).

Celecoxib is a potent inhibitor of prostaglandin synthesis as demonstrated by *in vitro* assay (Zhang *et al.*, 1997). Based on human recombinant-enzyme assay, celecoxib is considered to be 375 times more selective for COX-2 than COX-1 (Capone *et al.*, 2007). Werner *et al.*, (2002) studied the pharmacokinetics of celecoxib after a single dose of 200 mg in twelve human volunteers, one of whom had a CYP2C9, poor metabolizer genotype. In this study the authors have reported a t_{\max} of 2.9 hours, C_{\max} of 842 $\mu\text{g/L}$ and the AUC from time zero to infinity to be 6246 $\mu\text{g/hour/litre}$. The oral bioavailability of celecoxib is low (about 20-40%), due to its poor solubility in aqueous medium (Babu *et al.*, 2002b). Peak plasma concentrations are attained within 3 hours of an oral dose. The pharmacokinetics of celecoxib is linear over the clinical therapeutic dose range with AUC and C_{\max} increasing proportional to the dose (Paulson *et al.*, 2000). Celecoxib is extensively distributed in the body, indicated by its large apparent volume of distribution.

Paulson *et al.*, (2001) studying the effect of food on the absorption of celecoxib reported that a high fat meal delayed absorption by approximately 1- 2 hour and thus increasing the total absorption of celecoxib by 10-20% and a steady state plasma concentration attained within 5 days. Celecoxib is highly protein bound and extensively distributed into tissues and is eliminated by hepatic metabolism with less than 1% is excreted as unchanged drug (Searle, 2011). Its high lipophilicity makes celecoxib sequestered in the body fat of the individual leading to slow and incomplete rate of absorption (Brune & Hinz, 2004).

Celecoxib is extensively metabolized by CYP2C9 and to a lesser extent by CYP3A4 (Kirchheiner *et al.*, 2003). Three metabolites been reported to be formed namely hydroxylated celecoxib, carboxy celecoxib and its corresponding glucuronide. All three

metabolites are inactive and after a single oral dose of celecoxib, 57% are excreted in faeces and 27% in urine (Tang *et al.*, 2000).

It has been found that 35% of Caucasians are poor metabolisers of celecoxib due to the CYP 2C9 gene polymorphism resulting in reduced enzyme activity. These patients have CYP2C9*1 or CYP2C9*2 gene variants (Lee *et al.*, 2002). Stempak (2005) in a study with paediatric patients genotyped CYP2C9*3 has found the AUC was higher by 10-folds. Thus, there is an elevated risk such as cardio vascular side effects associated with celecoxib administration due to CYP2C9 variants. There are also significant differences between paediatric and adults with respect to celecoxib disposition and consideration should be taken in the dosing schedules (Stempak *et al.*, 2005).

Paulson *et al.*, (2000) have reported a gender differences in the clearance of celecoxib in rats has been reported by. Their data show that male rats eliminate the parent drug faster from plasma compared to female rats. Thus, female rats have a greater exposure to the drug than the male and they show marked difference in celecoxib pharmacokinetics. Emami *et al.*, (2008) report that following administration of 200 mg of celecoxib to 12 healthy volunteers, the C_{max} was 450 ng/ml, the time required to reach a maximum in plasma (t_{max}) was 1.87 hours and the terminal half life was approximately 12 hours.

An upper gastrointestinal tolerability trial with patients receiving celecoxib from a low dose of 50 mg to a supratherapeutic dose of 400 mg twice daily was compared with naproxen 500 mg twice daily dose and placebo. This study monitored the incidence and time until moderate to severe pain, dyspepsia and nausea occurred and they concluded that the upper GI tolerability of celecoxib was much superior to naproxen (Bensen *et al.*, 2000).

A twelve week study to determine celecoxib (200 mg) versus diclofenac (50 mg) thrice a day at 43 centres in United Kingdom, reported that celecoxib was noninferior to diclofenac in treating arthritis pain after joint replacement (Emery *et al.*, 2008). Moreover a supra therapeutic dosage of 1200 mg/day did not have any significant effect on platelet function or bleeding time in healthy volunteers (Leese *et al.*, 2000). Gastrointestinal complications are the most important adverse effects of NSAIDs. In their study, Patterson *et al.*, (1999) explored the incidence of allergic reactions with celecoxib in patients who were hypersensitive to sulfonamide-containing medications. They found that celecoxib does not inhibit the COX-1 enzyme. At therapeutic doses, celecoxib is equally effective as any other commonly used NSAIDs such as ibuprofen, naproxen, ketoprofen and diclofenac, but with little or no adverse effect on either the gastrointestinal tract or kidneys (Simon *et al.*, 2002). In a comparative study of celecoxib and ibuprofen, Al Sukhun *et al.*, (2012) recently confirmed that celecoxib has superior analgesic effect after postoperative pain following surgery.

Endoscopy in patients with arthritis has shown that 12-13 weeks of 200 or 400 mg/day celecoxib administration caused fewer incidences of gastrointestinal ulcers when compared with ibuprofen (2400 mg/day). Moreover, the results reveal that a greater number of patients discontinued the treatment due to the adverse event with ibuprofen (Hawkey *et al.*, 2004) or naproxen (1000mg/day) (Goldstein *et al.*, 2008).

The SUCCESS-I study involved a large population of 13,274 patients over a 12 week period and the data demonstrated that celecoxib at 200 mg or 400 mg/day had significantly lower incidence of ulcers compared with diclofenac (100 mg/day) or naproxen (1000 mg/day) (Singh *et al.*, 2001). Cryer *et al.*, (2011) in their GI-REASONS study which took place for a period of 6 months demonstrated a significantly higher

incidence of GI events with traditional NSAIDs such as meloxicam, diclofenac, nabumetone and naproxen when compared with celecoxib.

In the CONDOR (Celecoxib versus omeperazole and diclofenac in patients with osteoarthritis and rheumatoid arthritis) study employing 4484 patients demonstrated that celecoxib for 6 months at 400 mg/day had significantly lower incidence of GI events than diclofenac (150 mg/day) and omeperazole concentrations taken together (Chan *et al.*, 2010).

However, the CLASS (Celecoxib Long Time Arthritis Safety Study) trial involving 8059 patients and using a supratherapeutic dose of 800 mg/day, showed that celecoxib was not significantly less than other NSAIDs (ibuprofen 2400 mg/day or diclofenac 150 mg/day) in inducing gastric ulcers. When combined with low dose aspirin, celecoxib had ulcer complications lower than other NSAIDs (Silverstein *et al.*, 2000a).

Figure 1.8 shows the schematic pathway of the metabolism of celecoxib in humans. The different chemical structures show that celecoxib undergoes extensive hepatic metabolism in humans predominantly by the cytochrome P450 (CYP) 2C9 isoenzyme, with less than 3% excreted unchanged in urine and faeces (Karim *et al.*, 1997). Three metabolites of celecoxib have been identified. Carboxyl metabolite formed by the complete oxidation of the methyl moiety of celecoxib is the major metabolite (M2). Partial oxidation of the methyl moiety followed by hydroxylation forms a second metabolite (M3) and glucuronidation forms the third metabolite of celecoxib (M1). The elimination half-life is approximately 12 hrs and plasma clearance is about 450 ml/min (Patterson *et al.*, 1999). The pharmacokinetics of celecoxib has not been studied in horses.

1.10.3. Pharmacokinetics and pharmacodynamics of etoricoxib

Etoricoxib, 5-chloro-3-(4-methanesulfonylphenyl)-6'-methyl-[2,3']-bipyridinyl is an NSAID with an empirical formula $C_{18}H_{15}ClN_2O_2S$ and a molecular weight of 358.84 (Chauhan *et al.*, 2005). Etoricoxib is approved as an oral medication in Europe for the treatment of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis (Anonymous, 2013). It is a COX-2 inhibitor producing dose dependant inhibition of COX-2 across the therapeutic dose range without inhibiting COX-1 (Dallob *et al.*, 2003). Etoricoxib displays linear pharmacokinetics since it is well absorbed after oral administration with a C_{max} reaching in approximately 1 hour in a fasting state (Schwartz *et al.*, 2008). The bioavailability is almost 100% and absorption rate is slow with the presence of food, but this does not disturb the extent of absorption (Agrawal *et al.*, 2002). The C_{max} and AUC from time 0 to 24 hours were 3.6 $\mu\text{g/ml}$ and 37.8 $\mu\text{g/hr/ml}$ after administration of 120 mg of etoricoxib to healthy subjects respectively (Agrawal *et al.*, 2003a).

Etoricoxib has been extensively studied in humans to determine its absorption, distribution, metabolism and excretion. Moreover, Riendeau *et al.*, (2001) found that etoricoxib selectively inhibited COX-2 in an *in vitro* human blood assay with an IC_{50} of $1.1 \pm 0.1 \mu\text{M}$ for COX-2 (LPS induced prostaglandin E2 synthesis), compared with an IC_{50} value of $116 \pm 8 \mu\text{M}$ for COX-1 (serum thromboxane B2 generation after clotting of the blood).

Etoricoxib is well absorbed after oral administration in humans and peak plasma concentrations are achieved within 1 hour. The absolute bioavailability is $\approx 83\%$ and averaged $\approx 101\%$ in a study of its bioavailability after fasting and a high-fat meal, with single and multiple doses (Agrawal *et al.*, 2003a). Pharmacokinetics of etoricoxib is

linear at clinical relevant doses. Agrawal et al., (2001) suggest that the pharmacological half-life and pharmacological response support once-a-day oral dosing of the drug. A 12-week study has found that etoricoxib (90 mg/once daily) was effective in treating rheumatoid arthritis than the traditional NSAID naproxen (500 mg twice daily) (Matsumoto *et al.*, 2007). In a recent study, Clarke et al., (2012) conclude that a single oral dose of 120 mg of etoricoxib to humans, can produce good quality pain relief after surgery and an amount of 120 mg is as effective as, or better than, other commonly used analgesics.

The results of an *in vitro* study with human liver microsomes has demonstrated that etoricoxib is metabolized via 6'-methyl hydroxylation, 1'-N-oxidation and 6'-carboxy etoricoxib (Chauret *et al.*, 2001). A schematic pathway of the metabolism of etoricoxib in healthy human volunteers is shown in Figure 1.9. which shows that etoricoxib undergoes hepatic metabolism to form 6'-hydroxymethyl etoricoxib, 6'-carboxy etoricoxib, etoricoxib-1'-N-oxide, 6'-hydroxymethyl etoricoxib-1'-N-oxide and glucuronide of 6'-hydroxymethyl etoricoxib in urine.

Etoricoxib is extensively metabolized by CYP3A4 enzyme and to a lesser extent by CYP2D6, CYP2C9, CYP1A2 and CYP2C19 in humans resulting in the formation of five metabolites. None of the metabolite is pharmacologically active. The main metabolites are the 6'-carboxylic acid derivative followed by the 6'-hydroxy methyl derivative, which are mostly excreted renally (Kassahun *et al.*, 2001). Terminal half life is approximately 22 hours, suggesting a once daily dose. About 70% of the drug is excreted in urine and 20% in faeces, mostly as metabolites (Rodrigues *et al.*, 2003). Age and gender do not have a significant effect on the pharmacokinetics however, etoricoxib

is contraindicated in patients with renal impairment (Agrawal *et al.*, 2004).

Discussions on the cardiovascular safety of all COX-2 selective NSAIDS are on for a long time. In 2004 rofecoxib was voluntarily withdrawn from the market after the APPROVE (Adenomatous Polyp Prevention on Vioxx) trial, showing an increased risk of myocardial infarction and stroke (Bresalier *et al.*, 2005). Similar trials with celecoxib and valdecoxib suggest that all COX-2 inhibitors bear a risk of cardiovascular events (Andersohn *et al.*, 2006). The MEDAL (Multinational Etoricoxib and Diclofenac Arthritis Long term) study compared the effect of etoricoxib (60 mg and 90 mg) with of diclofenac (150 mg), once daily on 34,701 patients with osteoarthritis and rheumatoid arthritis. The study reported that the rates of cardiovascular events in patients with arthritis on etoricoxib are similar to those patients on the traditional NSAID diclofenac with long term use of these drugs (Cannon *et al.*, 2006). Similarly, Etoricoxib versus diclofenac sodium on gastrointestinal tolerability and effectiveness (EDGE) trial studied a total of 7111 patients with osteoarthritis receiving 90 mg of etoricoxib once daily and 50 mg of diclofenac thrice a day. The study concluded that etoricoxib was associated with significantly better GI tolerability compared to diclofenac (Baraf *et al.*, 2004). Peleso *et al.*, (2011) in their randomized double blind trial with 387 patients receiving etoricoxib at 90 mg and 120 mg versus 1000 mg of naproxen conclude that for every 2 patients treated with etoricoxib, 1 achieved meaningful improvement compared to 1 in every 3 patients treated with naproxen. These initial studies have clearly shown that the two coxibs have been investigated thoroughly in humans subjects examining the pharmacodynamic and pharmacokinetic properties compared to other NSAIDs. However the literature on the use of these two coxibs on large animals is limited.

1.10.4. Method of Analysis of Celecoxib

Development of sensitive and specific analytical techniques for the determination of celecoxib in biological samples is of paramount importance for any pharmacokinetics study. High performance liquid chromatography (HPLC) with either ultraviolet (UV) or fluorescence detection has been most widely used in the detection of celecoxib (Zarghi *et al.*, 2006b). A HPLC method with solid phase extraction of celecoxib in rats in either the presence or absence of inflammation has been reported (Guirguis *et al.*, 2001;Guirguis *et al.*, 2001). In addition, (Srinivasu *et al.*, 2000) developed a LC method for the quantitative determination of celecoxib in bulk drug and pharmaceutical doses.

Celecoxib and its processed related impurities were determined using reversed phase HPLC with UV detection (Rao *et al.*, 2006) . Furthermore, Rose *et al.*, (2000) described a HPLC method for the determination of celecoxib in human plasma. Briefly, they used a volume of 1.0 ml of plasma which was first deproteinated using acetonitrile, followed by solid phase extraction on C₁₈ cartridges employing by HPLC with UV detection. The assay was linear in the concentration range of 25-2000 ng/ml.

A liquid chromatography-tandem mass spectrometric quantification of celecoxib in human plasma and rat was also developed by (Brautigam *et al.*, 2001). Celecoxib and the internal standard were extracted from plasma by solid-phase extraction with a C₁₈ cartridge. The mobile phase consisted of a mixture of acetonitrile-water-ammonium hydroxide solution 25% (85:25:0.1) and the detection was by AB Sciex API 3000 (Applied Biosystems) mass spectrometer. The limit of quantification for celecoxib in human plasma was shown to be about 0.25 ng/ml.

Subsequently, Abdel-Hamid *et al.*, (2001) developed a robust, highly reliable and reproducible liquid chromatographic-mass spectrometric assay for celecoxib in human

plasma using sulindac as an internal standard. The method involved extraction of the analyte with ethyl acetate and chromatography on a C₁₈ column using acetonitrile-1% acetic acid solution (4:1) as the mobile phase. The mass spectrometer was programmed in the single ion monitoring mode to detect the positive ions for celecoxib and sulindac at m/z 382 and 357, respectively.

Werner *et al.*, (2002) developed a reversed-phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionization (APCI) to study the pharmacokinetic profile of celecoxib following administration of a single oral dose (200 mg) to 12 healthy human volunteers. Similarly, Stormer *et al.*, (2003) developed a reversed-phase liquid chromatography with ultraviolet absorbance detection for the determination of celecoxib, hydroxycelecoxib and carboxycelecoxib.

A simple and rapid HPLC method for the determination of celecoxib in human plasma for pharmacokinetic application was later developed and validated by (Zarghi *et al.*, 2006a). The limit of quantification was 10 ng/ml and the assay enabled the measurement of celecoxib for therapeutic drug monitoring. The metabolism of celecoxib in rabbits with combined LC/MS/MS using precursor ion, product ion scans and constant neutral loss of m/z 176 yielded three phase I and four phase II metabolites (Zhang *et al.*, 2000). A simplified solid phase extraction procedure, eliminating protein precipitation by using a poly(divinylbenzene-co-N-vinylpyrrolidone) sorbent was also developed using rat plasma (Guermouche & Gharbi, 2004).

1.10.5. Methods of Analysis of etoricoxib

Being the latest molecule, very few HPLC methods have been reported for the determination of etoricoxib in human plasma. Mathews *et al.*, (2004) developed an

analytical method for the determination of etoricoxib in human plasma and urine using solid phase extraction followed by HPLC with photochemical derivatization - fluorescence detection (260 nm) using one of the structural analogues as internal standard. The limit of quantification was found to be 5 ng/ml.

Rose et al., (2002), using a stable isotope of etoricoxib as an internal standard, developed a LC-MS-MS method with atmospheric pressure chemical ionization (APCI) and this was validated over a concentration range of 0.2 to 250 ng/ml. Later, Brautigam et al., (2003), using phenazone as the internal standard, validated a liquid chromatography-tandem mass spectrometric method for the determination of the cyclooxygenase inhibitor etoricoxib in human plasma. The limit of quantification was about 2 ng/ml. A reversed phase HPLC with photodiode array detection at 234 nm, over a linear concentration range of 20 – 1500 ng/ml, was developed by (Brum Junior *et al.*, 2006). The most recent method to quantify etoricoxib utilizes capillary zone electrophoresis using photodiode array at 234 nm and this method was developed by (Dalmora *et al.*, 2008). This method was able to detect three photo degradation product and one acid hydrolysis product of etoricoxib (Matthews *et al.*, 2004). Similarly, Werner et al., (2005) determined levels of etoricoxib in human plasma using LC coupled with APCI MS/MS after performing a liquid-liquid extraction.

Two analytical methods for the determination of etoricoxib in bulk drug formulation were previously reported. One method used HPLC with UV detection while the other employed UV spectrophotometry at 284 nm (Shakya & Khalaft, 2007).

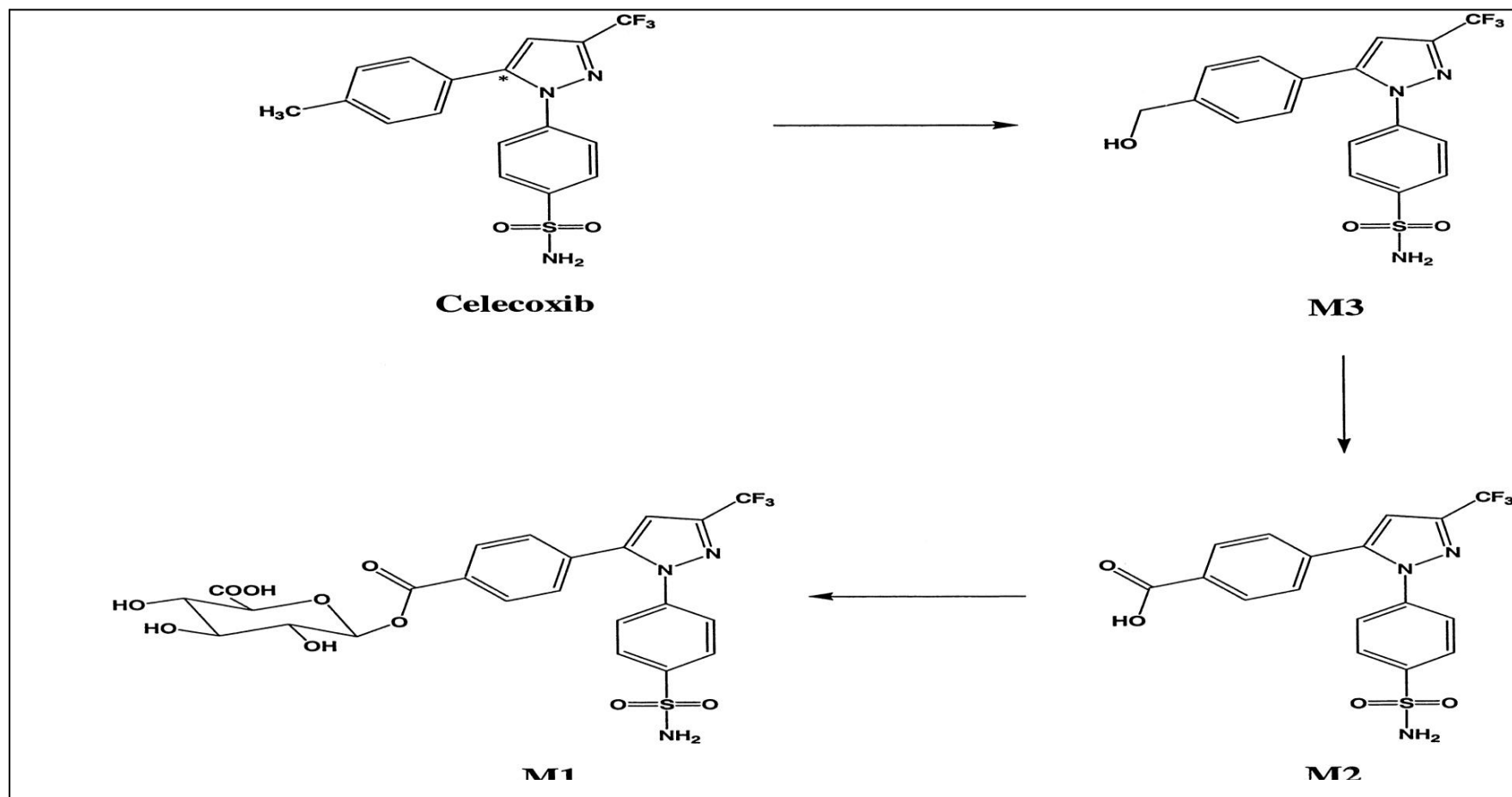


Figure.1.8: Metabolic pathway for celecoxib in humans (Taken from Paulson et al., 2000a).

1.11. Structure and function of the GI tract and the kidney in drug metabolism

Horse is a herbivore and is highly specialised for a plant diet with special dentition pattern with absence of canines. It has six pairs of incisors, since plant food has to be thoroughly ground before the digestive enzymes act upon it. The gastrointestinal tract of the horse serves as an important part for digestion and absorption of nutrients and also to detoxify xenobiotics. The size of the stomach is small, has a volume varying from 8-15 relative to its size, weight and volume consumed. The small intestine is of 22-25 metres and the large intestine is 7.5-8 metres in length. The small intestine is divided into duodenum, the jejunum and the ileum. The digestive products of the pancreas and the liver reach the intestine where together with the secreted digestive enzymes begin the process of digestion. The majority of digestion takes place in the first half of the small intestine. The mucosal layer of the small intestine has numerous absorptive cells - the enterocytes that secrete the digestive enzymes required for digestion and absorption of nutrients. The pH inside of the duodenum of the small intestine is usually less than 6 and about 7.5 in the jejunum (Merritt, 1999). The large intestine is divided into cecum and colon which are larger in size and rich in microbes compared to other species of mammals, primarily to digest cellulose which are not able to be digested by mammalian enzymes (Aspinall, 2009).

Generally, the GI tract plays an important role in food digestion and drug metabolism. The gut contains numerous proteolytic, lipid and carbohydrate enzyme which can break down complex molecules such as proteins, lipids and carbohydrates. In addition hydrochloric acid secreted by the stomach and bacteria in the GI tract facilitate the metabolism of other complex food and drugs. In general, most drugs consumed orally are either absorbed into the bloodstream, directly metabolized in the GI tract or excreted as a whole product with stool.

The liver is the largest glandular organ and has many functions vital for survival such as carbohydrate metabolism, fat metabolism, protein metabolism, storage and detoxification (Remmer, 1970). The liver has dual blood supply, comprising of the hepatic artery which oxygenates the organs from the aorta, and the portal vein carries the blood from the intestine. The portal vein of the liver filters and detoxifies the blood coming from the gastrointestinal tract. The liver is made up of cells called the hepatocyte which secrete bile acids and bilirubin which is emptied into the duodenum. Interestingly horses do not have a gall bladder to store bile. (Thomas P.Colville, 2002).

The drugs consumed by animals are absorbed directly into the blood stream from the GI tract as by products, due to metabolism. The parent drug and its metabolites are taken to the liver where they are further broken down by enzymes. The drug and its metabolites can be excreted in the stool or taken back to the kidneys where they are filtered into the urine and subsequently excreted.

The kidneys play a major part in the excretion and removal of xenobiotics and soluble waste products from the blood and excess water from the body. They maintain the body's water and electrolyte balance stimulate red blood cell production through erythropoiesis. The kidneys of the horse weigh about 700 grams each with the left kidney bean shaped and the right kidney heart shaped. Oxygenated blood enters the kidney through the renal artery and exits via the renal vein. The functional unit of the kidney is called the nephrons and the equine kidney has approximately 1.5 - 2.5 million nephrons. The nephrons form the urine and it consists of a capillary network, glomerules and a tubular system. The glomerules capillaries are surrounded by Bowman's capsule. The glomerules filter the urine and ensure large proteins such as the blood cells do not get filtered into the urine.

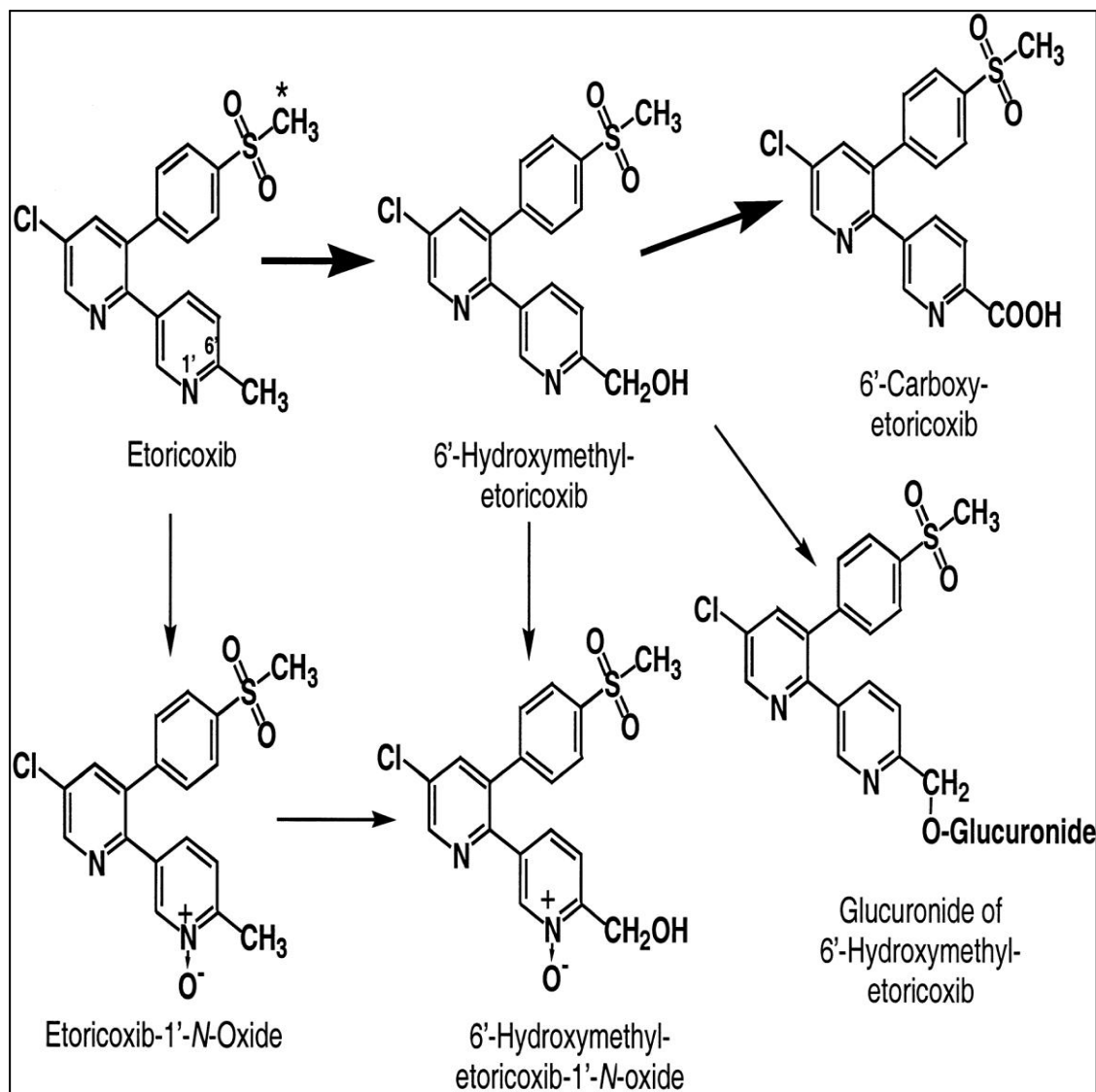


Figure 1.9: Metabolic pathways of etoricoxib in healthy human volunteers (Taken from Rodrigues et al., 2003).

The process of biotransformation of drugs and other xenobiotics, with large alterations to the drug molecule mostly happens in the liver and kidneys due of its large blood supply and the presence of drug metabolizing enzymes. In addition drug metabolizing enzymes present in the mucosal lining of the gastrointestinal tract probably contribute to the first pass metabolism of orally administered drugs.

1.12. The metabolism and fate of drugs in the body

Knowledge about the factors which determine the fate of drugs in biological systems of horse is essential in the field of forensic drug testing, forensic pharmacology and especially in doping. Correct drug dosing is of paramount importance for the welfare of the horse and moreover, to limit the use of performance enhancing drug in sports (Kietzmann & Due, 2009).

Horses, like humans and other animals, have complex physiological systems to disintegrate and to detoxify foreign chemicals that enter into their body. In order for drugs to exert their effect, in either in doping or in treatment, they need to achieve adequate concentration in the body or in the location of inflammation. This in turn depends upon absorption, distribution, metabolism and excretion, frequently abbreviated as ADME (Poulin & Haddad, 2012).

Absorption is the movement of drug from the site of administration to the blood stream. Distribution refers to the movement of the drug from the blood stream to the tissues. Metabolic reactions typically convert drugs into more hydrophilic form, making them more easily excreted by the body.. Compounds, including drugs and their metabolites are usually excreted from the body mainly by the renal and bile systems (Chu & Nomeir, 2006).

Oral administration is a convenient way to deliver a drug (Nuno Martinho, 2011). The major site for absorption of the drug in the body of an animal is the gastrointestinal tract. It is affected by stomach emptying and intestinal mobility, the environment in the intestinal lumen and the metabolizing enzymes present in the intestinal mucosa, lining and the liver (Kumar & Clark, 2009; Kumar & Clark, 2009). Drugs, by active and passive mechanisms, permeate the intestinal wall. As the drug reaches the blood stream, the cardiovascular system helps in the distribution of the drug molecule throughout the biological systems. Specialized proteins carry the drug from the intestine to the blood stream during an active mechanism. Hydrophilic drugs are passed on to cells through intercellular spaces via passive mechanism (Levine, 1978).

Metabolism is a process in which xenobiotics, (eg. drugs) are made more hydrophilic by endogenous enzymes, to be easily excreted (Alavijeh *et al.*, 2005). The liver and the intestines are the major sites of metabolism for an orally administered drug. Drug metabolism is normally divided into two phases, phase I and phase II. The chemical reactions that are associated with these phases are given in table 1.4.

Oxidation, hydrolysis and reduction are the most common phase I reactions, catalyzed by cytochrome P450 (CYP) enzymes in the liver. Glucuronidation and sulphation are the commonest phase II reactions for haepatically cleared drugs (Guengerich, 2001). Understanding the mechanism of action of a drug, the dosage, dosage interval and excretion profile are important parameters to be borne in mind in the field of horse racing as it is important to administer the correct dosage and to put the horse in top form to compete in a race. In order to assess these, usually different pharmacokinetic (PK) parameters are used as important scientific tools. The goal of these parameters is to obtain maximal efficacy in treated animals.

Table 1.4: Reaction classes of Phase I and Phase II metabolism

Phase I	Phase II
Oxidation	Glucuronidation
Reduction	Sulfation
Hydrolysis	Methylation
Hydration	Acetylation
Isomerisation	Glutathione conjugation
Dithioacetylation	

Bioavailability (F) is a key pharmacokinetic parameter and it refers to the rate and extent to which an active substance is absorbed from its pharmaceutical form and becomes available at the site of action (Toutain & Bousquet-Melou, 2004a). Several factors can influence the oral bioavailability of a drug. These include solubility, lipophilicity, stability and ionization, together with the several processes such as physiological, biochemical and pathological processes that occur in the lumen of the intestines. The fraction of drug that escapes the intestinal metabolism undergoes metabolism in the liver before reaching the systemic circulation (Routledge & Shand, 1979).

Clearance (Cl), Volume of distribution (V_d), and half life ($t_{1/2}$) are other important PK parameters, which are often used to study the fate of a drug in the body (URSO *et al.*, 2002). The ability of the body to eliminate the drug per unit time is termed as clearance. The time required for the plasma concentration of the drug to be scaled down to half, after reaching a pseudo equilibrium is termed as the half –life ($t_{1/2}$) of the drug. Terminal half-life is a key in clinical application to set dosing intervals, thereby avoiding drug

accumulation (Toutain & Bousquet-Melou, 2004b). V_d is the proportionality constant between total amount of drug in the body and plasma concentrations.

1.13. Liver enzymes and drug metabolism

The study of the metabolism of drugs by *in vivo* and *in vitro* methods gives vital information for laboratories involved in drug testing in sports (Teale & Houghton, 2010). Those drugs that are eliminated quickly, the detection of metabolites is more desirable. Moreover, *in vitro* experiments are particularly useful if a large number of biotransformation, especially in steroids, is possible thus leading to many possible metabolites. It would be difficult on the part of an analyst to identify the most appropriate *analyte* in controlling the misuse of therapeutic and performance enhancing drugs, without the information from *in vivo* and *in vitro* studies (Houghton & Maynard, 2010). Practical implications like the availability of animals for study and ethical issues including whether the drug or its metabolite(s) has toxicological profiles following administration are very important to address. These can be solved by employing both *in vivo* and *in vitro* studies. In the case of the latter, it is possible to use liver tissues or liver fractions (Thummel & Wilkinson, 1998). It has been revealed that compared to liver microsomes, liver tissues are especially useful in obtaining the complete *in vitro* metabolite profile, because it retains all the enzymes and cofactors essential for both phase I and phase II metabolism (Dogterom, 1993).

Drug metabolism is a process which involves the breakdown of a compound by enzymes in the body in order to make drugs and their metabolites more water-soluble and thus, more readily excreted in the urine or faeces (Slaughter & Edwards, 1995). The most common way of metabolizing drugs involves the alteration of functional groups on the parent molecule through a series of phase I and phase II reactions via the cytochrome P450 enzymes. These enzymes are most predominant in the liver but can

also be found in the intestines, lungs and other organs (Anon, 2000). Changes in the activity of these enzymes can lead to inactivation of an active compound, activation of an inactive compound, formation of an active metabolite for an active compound and in turn this can also lead to toxic effect of an active or inactive compound (Lin & Lu, 1997).

The advancement of bioanalytical and biochemical technologies has provided opportunities to study the *in vitro* metabolism and the *in vitro* metabolite profile generally reflect the *in vivo* metabolic pattern (Powis, 1989). The cytochrome P450 super family (CYP) is an enzyme group in humans critical for oxidation reactions in the phase I metabolism of xenobiotics (Anzenbacher, 2001). The name P450 was originally a description of a red pigment found in liver microsomes with an absorbance maximum at 450 nm in its reduced carbon-monoxide form (Omura & Sato, 1964). The cytochrome P-450 plays a significant role in the metabolism of more than half of the drugs used today (Guengerich, 1995). Though expressed in several tissues, the liver and the small intestine are the primary sites of interest for these enzymes for metabolizing drugs and other xenobiotics (Krishna & Klotz, 1994). The number of CYP isoenzymes are constantly increasing and as of 2012 more than 18500 CYP isoenzymes have been found both in humans and animals (Nelson, 2013). The CYP enzymes are classified into families and subfamilies based on their amino acid sequence and enzymes sharing more than 40% sequence identity are grouped to the same family (Wang & Chou, 2010).

The CYP enzymes of the family 1,2 and 3 are known to be important for drug metabolism and it has been studied that the activity of the CYP enzymes vary between individuals which may be due to genetic factors, environmental factors or either induction or inhibition of the enzymes (Bozina *et al.*, 2009). Fink-Gremmels (2008) in his study reveal that large inter species and intra species differences prevail when

comparing enzyme activity in different animals. The study of CYP enzymes in animals help in creating model systems in drug development for humans.

1.14. Cytochrome P450 enzymes in horses

The knowledge about CYP enzymes in other species of animals is limited compared to human enzymes and it is nevertheless important since companion animals and animals for food production are treated with various drugs (Gusson *et al.*, 2006). It is often impractical to study *in vivo* drug administration to large animals such as horse, because of its availability for research purpose and the expense associated in maintaining them. Horse racing is a billion dollar industry and racing labs are constantly challenged with new compounds that have the potential for abuse. These new compounds might metabolize quickly and most often not present as an intact parent compound in urine or other biological matrix, thus making a search for the unknown metabolite of the compound more challenging (Nebbia *et al.*, 2003).

The human cytochrome P450 enzymes have been well characterized in the biotransformation and subsequent elimination of many xenobiotics. These characterization studies have given an understanding of the specific CYP enzyme involved in the metabolism of individual drugs. As in humans, better knowledge of equine enzyme system will allow the field to understand and predict drug interactions, alterations and the pharmacokinetics.

Members of the cytochrome P450 3A and 2C family are responsible for the metabolism of a vast majority of therapeutic substances in humans and similar information of the expression and activity of members of P450 family in horse is either limited or non extinct. Though horse liver microsomes have been used in drug metabolism studies, these studies have not provided information about the specific enzyme involved in metabolism (Chauret *et al.*, 1997).

Few years ago, Knych *et al.*, (2009) have cloned, sequenced, expressed and characterized an equine CYP450 orthologue, designated CYP2C92 and is shown to have sequence homology with other members of the CYP2C family in other species. Tyden *et al.*, (2004) investigated the gene expression of CYP 3A in the intestine and the liver in horse using real-time RT-PCR and showed that the CYP3A gene expression was the highest in the intestine than the liver. Thus, their study has highlighted that the intestine will metabolize a larger portion of all xenobiotics entering through the oral route with the CYP 3A enzymes than the liver in horses. Again very recently, Knych *et al.*, (2010) have cloned and sequenced three equine cytochrome P450 monooxygenases of the 3A family and designated them CYP3A89, CYP3A96 and CYP3A97. These new enzymes showed high degree of homology between the isoforms and shared a 75% identity to CYP3A4 of humans.

1.15. Metabolite profiling and identification *in vitro*

The aim of *in vitro* metabolite profiling is to tentatively predict the number of metabolites formed by various biotransformation reactions and abundances of the formed metabolites. Moreover, the nature of occurred biotransformation reactions and their sites in the molecule are elucidated, meaning identification of the chemical structures of the formed metabolites (Olavi Pelkonen, 2009).

Tang *et al.*, (2000) studied the *in vitro* microsomal metabolism of celecoxib in humans and showed that it forms one major metabolite, hydroxycelecoxib and no carboxy celecoxib was detected. Similarly, Wilhelmi *et al.*, (2009) investigated the *in vitro* drug metabolism of celecoxib using bovine liver microsomes and in their study they found carboxycelecoxib as the major metabolite.

Kassahun *et al.*, (2001) studied the role of human liver cytochrome P450A in the metabolism of etoricoxib and they showed that it is metabolized via 6'-methylhydroxylation when incubated with NADPH- fortified human liver microsomes resulting in only traces of 1-N oxide. In their study on etoricoxib with microsomes Chauret *et al.*, (2001) detected and structurally identified two metabolites, namely 6'-hydroxy methyl and 1-N oxide. Furthermore, when incubated with hepatocytes from dog, they identified a third metabolite of 30 mass units greater than the parent etoricoxib which was later characterized to be 6'-carboxy etoricoxib. They also noted a new metabolite with 192 mass units greater than the parent drug and this compound was identified as a glucuronic acid fraction of 6'-hydroxy methyl etoricoxib.

1.16. Gastrointestinal safety of COX-1 and COX-2 inhibitors in horses

COX-2 selective inhibitors are known to have better gastrointestinal (GI) safety profile compared to traditional NSAIDs providing either similar or enhanced anti-inflammatory effects compared to first generation coxibs (Deeks, 2002). Most commonly reported adverse side effects include nausea, dyspepsia, diarrhoea, abdominal pain and flatulence in humans (Farkouh *et al.*, 2004; Bombardier *et al.*, 2000). In a human study named Vioxx Gastrointestinal Outcome Research (VIGOR), Bombardier *et al.* (2000) compared rofecoxib with naproxen which was the first large-scale trial to provided evidence of lower incidence of GI events with COX-2 inhibitors.

Celecoxib Long-term Arthritis Safety Study (CLASS), compared celecoxib with traditional NSAIDs like diclofenac, and it reported lesser side effects of 0.76% versus 1.45% for diclofenac (Silverstein *et al.*, 2000a). The gastrointestinal adverse events were lower when compared with diclofenac in the Etoricoxib Versus Diclofenac Sodium Gastrointestinal Tolerability and Effectiveness (EDGE) trial (Baraf *et al.*, 2007). The Multinational Etoricoxib and Diclofenac Arthritis Long-term (MEDAL)

programme – the largest randomized trial also concluded that etoricoxib has significantly less common GI ulcers than diclofenac (Laine *et al.*, 2007).

In the racing industry, NSAIDs are widely used to treat inflammation and they help to mask lameness while reducing pain, swelling and inflammation, but they can also be very harmful to the racing horse (equine athlete). NSAIDs can contribute to GI ulcers, colic, renal and liver toxicity and also they can decrease a horse's health and racing performance.

Many drugs are used in horses and one of the oldest drug, aspirin, has been used for a long time for its anti-inflammatory and analgesic activity, but it has been discontinued because it was found to prolong bleeding time in horses for up to 48 hours (Cambridge *et al.*, 1991). Phenylbutazone was introduced into Veterinary Medicine in the early 1950s and it was also found to have analgesic, anti-inflammatory and antipyretic activities, which extend for more than 24 hours, due to the slow excretion of its oxyphenylbutazone metabolite (MacAllister *et al.*, 1993). Phenylbutazone, which is commonly called 'BUTE' by Veterinarians, causes haemorrhages and ulcers in the mouth, oesophagus and stomach. Phenylbutazone is mostly used to disguise lameness for the purpose of competitive racing (Shannon K.Reed *et al.*, 2006). In a study, Mc Connico *et al.* (2008) investigated the pathophysiological effect of phenylbutazone and they conclude that prolonged administration of BUTE can cause hypo-albuminemia and neutropenia. Dipyrone is another drug that has been used, but not well investigated in horses. High doses of dipyrone may result in abnormal blood cell production damaging the bone marrow of the horse (Rohner K & Demuth D, 1994). Flunixin meglumine is a very potent cyclooxygenase inhibitor which is in current use to treat horses. In these animals it helps to reduce a variety of painful and inflammatory conditions like colic,

eye injuries, respiratory diseases, general surgery, and musculoskeletal disorders (Stelio P.L.Luna *et al.*, 2007).

Lees & Higgins (1985) studied the pharmacology and therapeutic use of meclofenamic acid and found a decrease in blood protein concentration, together with mouth ulcers, edema, and depression. Ketoprofen is another NSAID, comparable to flunixin in its effect, but it was found to produce less toxic effects and ulcers than other COX-1 inhibitors (Jackman *et al.*, 1994).

Firocoxib is the only cyclo-oxygenase inhibitor developed for the use in animals till date for the treatment of osteoarthritis and inflammation. Cook *et al.* (2009) compared the effects of firocoxib and flunixin meglumine and they concluded from their study that both are effective analgesics, but firocoxib was more advantageous in horses during recovery from ischaemic intestinal injury. In another study, Back *et al.* (2009) used a force plate measurement technique to titrate the dosage of firocoxib required for effective treatment of lameness and they found that a dose of 0.1 mg/kg body weight was sufficiently enough to reduce pain in chronic lameness. In a more recent study, Marc Koene *et al.* (2010) employed 96 horses to validate the efficacy of firocoxib and they concluded that firocoxib was safer and did not alter the haematology and biochemical parameters in the horses. Deracoxib, a new COX-2 inhibitor drug, has been in the market quite recently, and it was specifically developed for pain relief in dogs (Bienhoff *et al.*, 2012). Adverse side effects such as damage to the kidneys has been reported in cats since these small animals cannot eliminate the drug (Khan & Mclean, 2012).

1.17. Liquid chromatography-Mass spectrometry

Combined liquid chromatography-mass spectrometry (LC/MS) has become the analytical work horse of choice for the quantitative and qualitative analysis in forensic/pharmaceutical drug testing due to its speed, resolution, selectivity and sensitivity (Kamel & Prakash, 2006;Prakash *et al.*, 2007). Combined LC/MS is not limited to only non volatile, polar and thermally labile compounds, but also for thermally stable, volatile and apolar materials which can also be detected by this instrument. Searchable databases like the WILEY or the NIST, are not available for LC/MS which makes it a difficult equipment for rapid screening of unknown compounds, but measurement of accurate mass of the compound makes it the ideal instrument in research and drug discovery. A mass spectrometer measures either the weight of a molecule or its fragment(s) and separates it according to its m/z (mass divided by charge) value. However, a HPLC coupled to a mass spectrometer offers advantage in the separation of polar compounds such as drug metabolites. Matrix components and co-eluting peaks, which usually cause ion suppression or signal enhancement, can be isolated by chromatography, thus increasing the resolution and sensitivity (Staack & Hopfgartner, 2007;Matuszewski *et al.*, 2003). Typical quantitative LC/MS applications in pharmaceutical research are *in vitro* ADME assays in drug permeability, metabolic stability and inhibition studies (Chu & Nomeir, 2006). Metabolite profiling and drug excretion study would be an example for qualitative LC/MS analysis in drug testing laboratory for reliable identification of metabolites in biological matrices.

1.18. Mass Spectrometry

Mass spectrometry (MS) is a technique where the mass weight of a molecule can be measured (Ho *et al.*, 2003). The resolved molecules being eluted by the liquid chromatography are transformed into gas phase ions, passed through electric and magnetic fields and the response of these trajectories are measured (Fenn *et al.*, 1989). Quadrupole mass spectrometers were first introduced in 1968 and the first triple quadrupole was developed at Michigan State University in the late 1970s (Yost & Enke, 1978).

Mass spectrometric detection has been the choice in many different field of application similar to the different kinds of analyzers developed over the years. Quadrupole (Q), triple quadrupole (QqQ), iontrap (IT) and quadrupole-time of flight mass analyzer (Q-TOF) have been designed and developed over years for accurate mass determination and structure elucidation of molecules. The functions of these mass analyzers are described below.

1.19. Triple Quadrupole Mass Spectrometry

A quadrupole MS consists of four perfect rods with each opposing rods connected with an oscillating electric field and a radio frequency between them, through which ions travel and are separated according to their m/z value (Skoog & Leary, 1992). A simplified schematic picture of a quadrupole is shown in figure 1.10. Ions released from the ion source are accelerated by an electric field in between the space and the four parallel rods with each opposite pairs of rods connected by an electric potential. One pair has a positive potential and the other is negative. Additionally, a radio frequency (RF) current is also applied on each pair of rods. Ions with specific m/z mass intervals oscillate along the central axis in a stable manner and reach the detector. Those masses

which are out of range or those masses that are unspecific oscillate undesirably. They then hit the rods, get neutralized and are subsequently lost (Herbert & Johnstone, 2003).

A triple quadrupole mass spectrometer (QqQ) has two quadrupoles (Q) and a connecting collision cell in between. The first quadrupole (Q1) acts as a mass filter which transmits the ions to the collision cell (q). The collision cell in most instruments is another quadrupole and rarely could be a hexapole or an octapole. The collision cell has a RF potential applied and also it has a supply of an inert collision gas such as argon to convert kinetic energy of the ions into internal energy. This results in bond breakage and dissociation of ions into small fragments, a process called collision-induced dissociation (CID). The collision cell accelerates the ions into the third quadrupole (Q3) which in turn analyses the fragments and sends them to the detector (Dass, 2007). Triple quadrupole instruments enable tandem mass spectrometry (MS/MS) in space and it can be operated in different scan modes as illustrated in Figure 1.11.

In the normal MS mode, either Q1 or Q3 quadrupole could be selected without the collision cell to obtain a full mass spectrum. In the product ion scans a specified m/z value which is selected in the Q1, passes on to q, where it undergoes CID with collision gas and the products are scanned in Q3. The precursor ions are selected in Q1 in precursor ion scan and the products are scanned in Q3. This is the opposite of product ion scan. A drug molecule during metabolism, adds on a mass charge of 176 Da or 80 Da depending on glucuronic acid or sulphate conjugation. In a neutral loss scan, both Q1 and Q3 are set to scan ions with a specified mass offset of 176 Da or 80 Da between them. Thus, the mass spectrum shows the precursor ion that loses the m/z of 176 Da or 80 Da in the collision cell and indicating the presence of glucuronic acid or sulphate conjugation within the sample.

Finally, excellent sensitivity and selectivity are achieved in selected reaction monitoring mode (SRM). In this process, one mass ion is filtered by Q1, fragmented in q, and the product fragments are allowed to pass through Q3 and to the detector. Since the fragmentation pattern is unique for each individual xenobiotic, molecules having the same precursor ions can be clearly separated from one another by Q3 product ions.

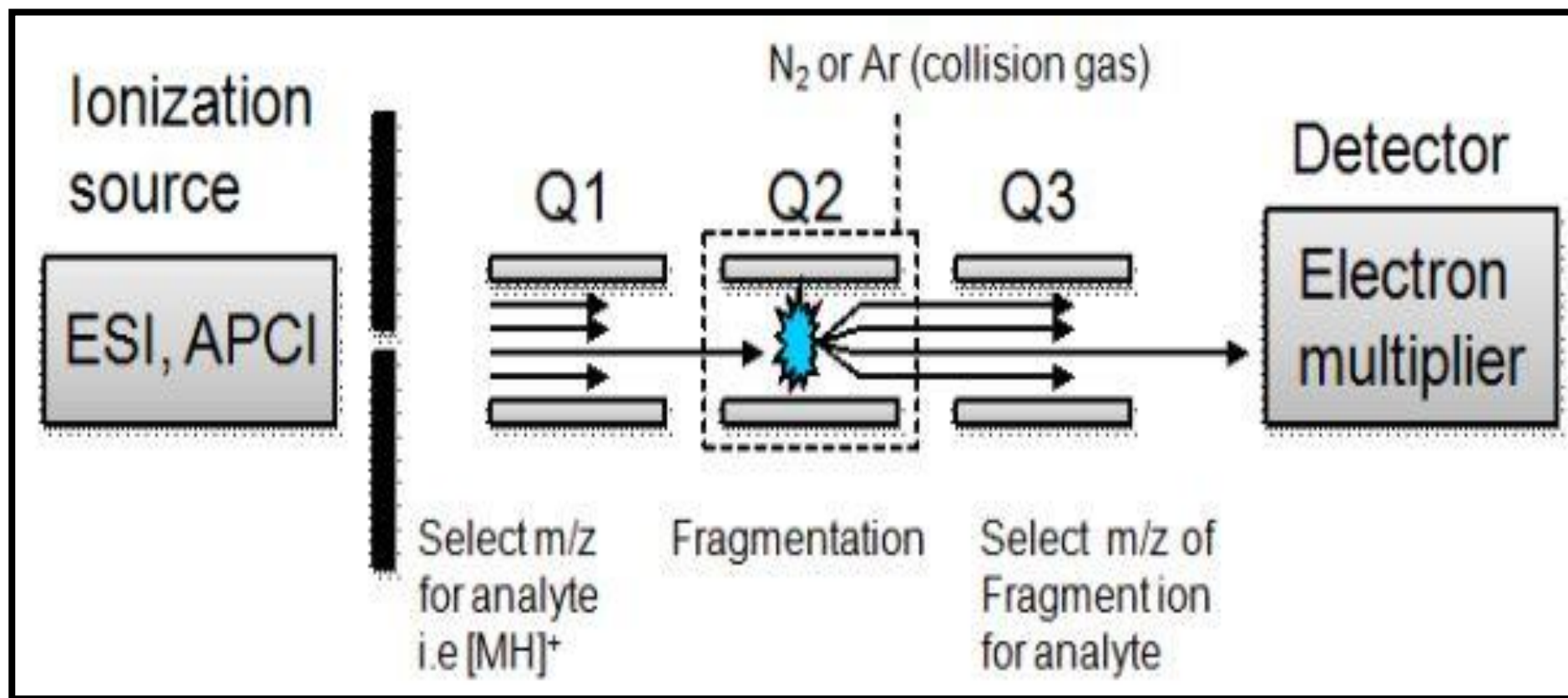


Figure 1.10: Schematic diagram of a triple stage quadrupole mass analyzer (Taken from Jinsong & Josh, 2012).

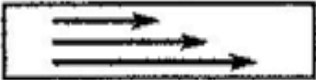
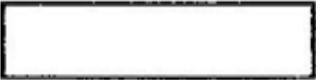
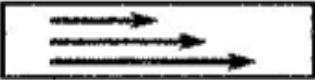
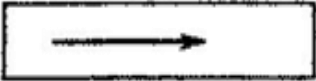
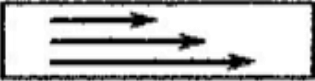
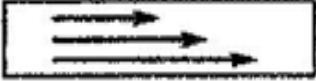
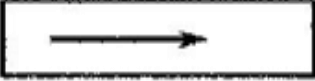
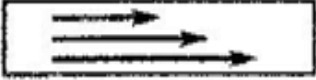
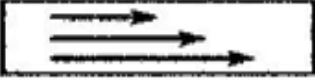
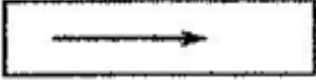
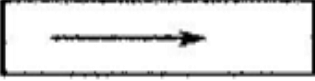
Mode of operation	Q1	q (Collision cell)	Q3
MS scan			
Product ion scan		CID	
Precursor ion scan		CID	
Neutral loss scan		CID	
Selected reaction monitoring		CID	

Figure.1.11: Schematic description of different scan modes that can be use in a triple quadrupole mass spectrometer (Taken from Thurman & Ferrer, 2002).

1.20. Working hypothesis:

In the horse racing industry it is sometimes very difficult to distinguish between doping and pain treatment relief of the animals employing NSIADs. Knowledge and understanding of the metabolism of some new c(Braeutigam *et al.*, 2003)oxib NSIADs including celecoxib and etoricoxib will help the racing industry, the race horse owners and the Equine Veterinarians in treating the horses effectively and to determine any side effects and more so, to distinguish any misuse of the drugs for racing gain.

1.21. Main Aims

The main aim of this study was to investigate any adverse effect each drug may have in the animals and furthermore to determine the fate, metabolism and elimination time of the two NSAIDs, celecoxib and etoricoxib in retired race horses and whether the drugs or their metabolites can produce adverse effects to the horses.

1.22. Specific Aims

The specific aims of this thesis were:

- To undertake a thorough literature search in the proposed area of study. This involved review of literature on NSAIDs, physiology and anatomy of the horse, pharmacokinetics and pharmacodynamics of commonly used NSAIDs in humans and in animal species. Literature search also involved the review of latest analytical techniques used in the detection of celecoxib and etoricoxib in blood, urine and faeces of human and other animal species such as dogs and monkeys.
- To study the pharmacokinetics effects of the two non-steroidal anti inflammatory drugs, etoricoxib and celecoxib in retired race horses. This part of

the study involved oral administration of the drug to the horses followed by the collection of blood, urine and stool samples. The pharmacokinetic parameters were analyzed using Microsoft excel and online pharmacokinetics software.

- To study and measure the different metabolites formed by the drugs administered and to analyze the routes of elimination for each drug, the time of elimination, the quantity of each and the mass of each metabolite. In this study, blood, urine and faeces were analyzed for the parent compound and its metabolites for each coxib over time using HPLC/MS/MS techniques.
- To use commercial horse liver microsomes *in vitro* to investigate the metabolism of etoricoxib and celecoxib. Each drug was incubated with horse liver microsomes overtime and the different samples were analyzed for the biotransformation of the parent drug to its corresponding metabolites.
- To study the metabolism of etoricoxib and celecoxib using fresh camel liver tissues. Each drug in known concentrations was incubated with camel liver slices overtime. Thereafter, both the parent drug and its metabolites were measured in samples using LC/MS/MS.
- To measure the levels of a number of blood-borne clinical biochemical parameters and ions (chloride, phosphate, Na^+ , K^+ , Mg^+ , Ca^{2+} , Fe^{2+} , Zn^{2+} , and Cu^{2+}) following oral administration of each coxib. Both Atomic absorption spectroscopy, haematology analyzer and biochemical auto analyzer were employed to measure the various parameters.
- To analyze the data statistically and to write up the PhD thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

Animals:- Six retired race horses (2 males, 2 females and 2 castrated males)

Equipment:- THERMO FINNIGAN LC/MS/MS , iCE 3000 series flame and Zeeman furnace Atomic Absorption Spectrometer (AAS), Zymark Rapid trace, Zymark TurboVap, SYSMEX XT 2000i from Thermo Scientific, USA. Haematology Analyzer and Hitachi 912 Auto-analyzer from ROCHE, France, VacElut vacuum manifold from VPS Systems, Germany.

Chemicals:- HPLC grade chemicals – methanol, ethyl acetate, hexane and acetonitrile (All purchased from FISHER Scientific, UK).
NADPH, β -glucuronidase from Helix pomatia, potassium dihydrogen phosphate, sodium phosphate and magnesium chloride (All purchased from SIGMA-Aldrich, USA).

Reagents:- 0.1% formic acid and acetic acid from Sigma Aldrich USA,.
500 mg, 3 ml C₁₈ cartridges from Agilent, USA., and Isolute HXC 200 mg (All purchased from BIOTAGE, Sweden. (Similarly, all biochemical and haematological kits were obtained from La Roche, France).

Drugs:- Celecoxib and zaleplon with greater than 99 % purity were obtained as A gift from IPCA Laboratories, Indore, India. Etoricoxib was also donated as a gift by M/s. Reddy Laboratories, Hyderabad, India.

Others:- Horse liver microsomes was purchased from Xenotech, Lenexa, Kansas, USA and camel liver tissues were obtained from the local abattoir in Dubai, UAE.

2.2. METHODS

2.2.1. Animals

Six adult horses (12 – 18 years) were used for the study. They were obtained as kind donations for the purpose of research after their successful term as race horses. It was not possible to obtain others due to excessive expenses in purchasing and upbringing the animals. All animals were housed in uniform air-conditioned stable barns (see figure 2.1.). Horses are large animals and they need sufficient space for movement and exercise. Each individual barn measured 16 x 20 feet with floor covered with wood shavings to absorb moisture. The horses were given regular walking exercise for 30 minutes every morning on a 50 meter long dirt track. Maintenance of the horses in air conditioned stalls called barns involved medical expenses, the cost of food, water and supplements together with appointment of stable attendants called horse grooms. The job of the grooms included feeding the horses, exercising them on a daily basis, cleaning the barns and collection of biological samples. The Central Veterinary Research Laboratory in Dubai, UAE had the facility in terms of space and funds to house 6 horses and thus they were used in the study.

Regular physical examination was performed on each horse by the Local Hospital Veterinary Doctor on arrival at the Institution and all the horses were found to be healthy. Their weights (mean \pm SD) were 474.1 \pm 21.7 kg (range 410-500 kg). The animals were fed twice a day with hay, alfalfa and grains and they all had free access to water. None of the horses had any disease history in their stable records. The study was approved by the Animal Ethics Committee of the Central Veterinary Research Laboratory in Dubai, UAE and by the Animal Ethics Committee of the University of Central Lancashire, Preston, United Kingdom.

2.2.2. Drug material

Etoricoxib, celecoxib and the internal standard zaleplon were donated by M/s r.Reddy's Laboratories Ltd. Hyderabad, India and Ipca Laboratories Limited, Indore, India. All had an assay purity of $\approx 99.8\%$. Horse liver microsomes were bought from Xenotech, USA and the fresh camel liver tissues were obtained from the local abattoir immediately after humane killing of the animal.

2.2.3. Treatment group and study protocol

Table 2.1: Details of the *in vivo* study design

Studies	Dosages	Study drugs	Wash-out period
I	1 mg/kg	Etoricoxib	2 weeks
II	0.5 mg/kg	Etoricoxib	2 weeks
III	2 mg/kg	Etoricoxib	2 weeks
IV	2 mg/kg	Celecoxib	2 weeks

2.2.4. Drug administration and sample collection

The study involved a single oral dose treatment of either etoricoxib at either 0.5 mg/kg, 1 mg/kg b wt, 2 mg/kg or celecoxib at 2 mg/kg b wt to six horses using a naso-gastric tube. The administration of each dose of the drug was done in the presence of a Clinical Veterinary Doctor. The drug was mixed in 250 ml of distilled drinking water and poured into the tube using a funnel. The funnel was rinsed with another 100 ml of drinking water to ensure complete delivery of the drug into the stomach. Feed was withheld to these horses 10 hours before and 6 hours after the administration but, water



Fig.2.1. A typical photograph showing the race horses housed in stable and employed in the study.

was made available at all times. A 18 gauge catheter was placed either into the right or left jugular vein for collection of blood samples. The study protocol was also approved by the Ethics Committees and the drug administration was performed by a qualified Veterinarian attached to the Equine Hospital, Dubai, UAE. Blood samples were collected into heparinised tubes at 0 (control), 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 24, 48, 72, 96, 120, 144, 168 hours, respectively. Samples were transported to the laboratory in ice cooled boxes within 5 minutes of collection. One portion of the each blood sample was subjected to haematology and biochemical studies, while the remaining sample was centrifuged, plasma separated and kept frozen at - 20°C until use for drug analysis. A custom made diaper was tied to the animal for the collection of urine and faeces was collected as nature delivered at the same period of time. Urine and faeces samples were collected whenever the animal disposed for seven days after administration and were pooled into morning and evening urine and faeces composites and stored at - 20°C until used for drug analysis.

2.3. Measurements of blood-borne biochemical and anion and cation parameters following oral administration of either etoricoxib or celecoxib

Baracho et al. (2009) using Sysmex K-800 previously studied the cardiovascular and haematological effects produced by chronic administration of etoricoxib in rats. This previously described method was employed in this study with minor modification. In this study an attempt was made to investigate the effect of the COX-2 selective inhibitors, etoricoxib and celecoxib on the haematological and biochemical parameters in samples of horse blood using Sysmex XT-2000i (KOBE, Japan) compared to untreated control samples. The instrument employed in the study to undertake the measurements was an automated haematology analyzer for animal blood. RBCs and platelet count were analyzed by the RBC detector of the equipment using Hydro Dynamic focusing method where cells were counted as they were forced to pass through

a small tunnel, causing disruptions in a laser light beam or electricity flow. The analysis of WBC was done by an optical detector block based on flow cytometry. Haemoglobin was estimated by the HBG detector based on the sodium lauryl sulfate (SLS) haemoglobin detection method.

Briefly, a volume of 4 ml of horse blood sample for each time point was submitted for haematological and biochemical analysis using the SYSMEX XT 2000i haematology analyzer. They included red blood cells (RBC), haemoglobin (Hb), packed cell volume (PCV), mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), platelets (PLT), white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS) and basophils (BASO). All values were expressed appropriately against the machine standards as %, g/l, mmol/l etc.

Similarly, a Hitachi 912 Auto-analyzer was used to estimate the different biochemical and clinical parameters in blood while the concentration of each cation in plasma samples was measured using an atomic absorption spectroscopy. The principle of the equipment method was mainly calorimetric and photometric detections. A small volume (4 ml) of each sample of blood was applied to either equipment which measured the concentrations of iron, creatinine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), Gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), creatinine (CREA), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), magnesium (Mg), calcium (Ca), phosphate (PHOS), sodium (Na), zinc (Zn), copper (Cu), potassium (K) and serum amyloid A (SAA) against each appropriate respective standard. All values were expressed appropriately against the machine standards as %, g/l, mmol/l etc. A typical chart showing the values of all blood borne parameters estimated in this study, both prior to administration and after administration of either celecoxib or etoricoxib, together with the normal reference

values for all haematological and biochemical parameters is shown in the Appendix (table 1).

2.4. Optimization of the method and extraction of etoricoxib from plasma

Braeutigam et al. (2003) previously described a detailed method both for the extraction and optimization of etoricoxib using solid phase extraction and analysis in human plasma by tandem mass spectrometry with electrospray ionisation. Similarly, Rodrigues et al. (2003) studied the effect of a single dose administration of etoricoxib and characterized its metabolites in plasma, urine and faeces in rabbit. These two methods were employed in this study for the extraction of etoricoxib and to study its metabolites in plasma, urine and faeces of the horses. In this method and in the present study, optimization of the extraction procedure was achieved by extracting positive control samples (10, 100 and 200 ng/ml) were spiked either into plasma, urine or faeces and extracted to study the selectivity, specificity, recovery, accuracy and precision. This initial method was repeated more than 25 times in order to achieve maximum optimization of the extraction procedure.

2.5. Preparation of etoricoxib stock solution and standards for plasma analysis

The stock solution of etoricoxib was prepared by weighing 1 mg of the reference material into a 10-ml volumetric flask and diluting it to volume with acetonitrile and deionised water (1:1, v/v) to obtain a concentration of 100 µg/ ml. The working solution for etoricoxib was prepared by diluting 100 µl of the stock standard to 100 ml into a 100 ml volumetric flask with deionised water to obtain a concentration of 100 ng/ml. The solution was stable for at least 6 months when stored under light-protected condition at 4 °C.

Internal standard was prepared by weighing 1 mg of zaleplon into a 100 ml volumetric flask and dissolving up to mark to get a concentration of 10 µg/ml with deionised water. Furthermore, a volume of 1 ml was pipetted into a 10 ml volumetric flask and made up to mark giving a concentration of 1 µg/ml solution of zaleplon.

About 100 ml of drug free plasma was collected from previously non-administered healthy horses, pooled, vortex-mixed and used for the preparation of calibrants. Plasma samples as calibrants were prepared at concentrations of 2, 5, 10, 20, 50, 100, 200, 500 ngs/ml by adding 20, 50, 100, 200, 500, 1,000, 2,000 and 5,000 µl of 100 ng/ml etoricoxib to 1 ml plasma. Zaleplon as internal standard (200 µl) was added to each. A plasma sample without etoricoxib added into it was used as '0' or negative control.

2.6. Extraction of etoricoxib from plasma samples

Briefly, following administration and collection, a volume of 1 ml of each plasma sample was extracted using Isolute HCX cartridges (3 ml, 200 mg). Prior to extraction, a volume of 200 µl of zaleplon (internal standard 1µg/ml) was added, vortex-mixed and centrifuged at 4000 g for 10 min. The extraction cartridges were placed on a 24-place vacuum manifold connected to a vacuum suction pump. The pressure was adjusted on the guage manually as requested. The cartridge is conditioned with 1 ml methanol followed by 1 ml phosphate buffer (pH 6.0). Calibrants and prepared samples were cautiously loaded onto the cartridge and allowed to pass through the cartridge under gravity or at low vacuum. The cartridge was washed with 1 ml phosphate buffer followed by 2 ml 1% acetic acid. Cartridge was dried under vacuum for 5 minutes. The analytes were eluted with ethyl acetate – ammonia (98:2). The solvent was evaporated under a stream of oxygen free nitrogen in a Zymark TurboVap at 56°C. The residue was reconstituted in 50 µl mobile phase. The negative control and the 2, 5, 10, 20, 50, 100, 200, 500 ngs/ml drug spiked plasma (calibrants) were run on an LC/MS/MS in selected

reaction monitoring mode. The area of the drug divided by the area of the internal standard was calculated and a plot of a standard graph curve, abundance verses concentration was plotted. A typical standard curve with real calculations is shown in the Appendix (table 2).

2.7. Etoricoxib extraction recovery, assay precision and accuracy

The extraction recovery of etoricoxib from plasma was evaluated in triplicates at three concentrations (10, 100 and 200 ng/ml) by comparing the peak areas of etoricoxib and zaleplon to the peak areas of corresponding compounds in samples prepared by spiking extracted drug free plasma with the same amount of compound.

Within-day accuracy and precision were evaluated by analysis of quality controls at concentrations of 10, 100 and 200 ng/ml (n=5 at each concentration) on the same day. The same experiment was repeated on three different days to assess the between-day accuracy and precision. Accuracy was calculated as the percentage ratio of measured concentration to nominal concentration. Precision was expressed as the coefficient of variation. The lower limit of quantification (LLOQ) of the assay was also determined during this process.

2.8. Extraction of etoricoxib and metabolites from urine samples

Samples 0 (control), 1 to 12, 12- to 24, 24- to 36, 36- to 48, 48- to 60, 60- to 72, 72- to 84, 84- to 96, 96- to 108, 108- to 120, 120- to 132, 132- to 144, 144- to 156, 156- to 168 hours collection intervals were thawed at room temperature and mixed thoroughly using a rota-mixer. Thereafter, a volume of 10 ml of urine from each time interval was adjusted to pH 5.2. A volume of 100 µl of β -glucuronidase solution (10,000 Fishman units, raw extract from *Helix pomatia*, (SIGMA ALDRICH) was added to each tube and incubated overnight at 37°C. in an incubator.

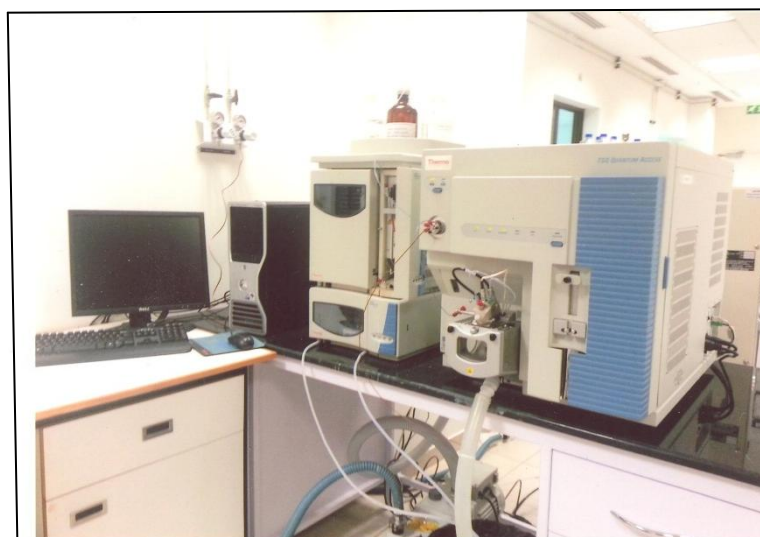


Figure 2.2 Photograph showing the THERMO FINNIGAN TSQ ACCESS
LC/MS/MS

After incubation, the samples were adjusted to pH 6.2, centrifuged at 4000 g for 20 minutes. A Zymark Rapidtrace solid phase sample extraction module was used to extract urine samples. Isolute HXC cartridge was sequentially preconditioned with methanol and 0.1 M phosphate buffer (pH 6.0). A volume of 5 ml of the urine sample was loaded to the cartridge at a rate of 0.02 ml/sec. The cartridge was then washed with 1 ml of 0.1M phosphate buffer (pH 6.0) followed by 1ml of 1M acetic acid. Thereafter, the extraction cartridge was dried under oxygen free nitrogen (OFN) for 4 minutes and eluted with 5 ml of freshly prepared ethylacetate : ammonia (98:2). The eluant was then transferred to a clean kimble tube and dried under OFN at 56°C.

2.9. Extraction of etoricoxib from faeces

Faeces samples were thawed at room temperature, an amount of 10 grams of the sample was accurately weighed and dissolved in 10 ml distilled water and thoroughly vortex-mixed for 10 minutes and then centrifuged at 5000 g for 20 minutes. A volume of 10 ml of the clear liquid was adjusted to pH 5.0 and 100 µl of β-glucuronidase solution (10,000 Fishman units, raw extract from *Helix pomatia*, SIGMA ALDRICH) was added to each tube and incubated overnight at 37°C. in an incubator. The pH was adjusted to pH 6.2 and drugs were extracted by solid phase extraction using ISOLUTE HXC cartridges. Zaleplon was used as an internal standard.

2.10. Determination of etoricoxib concentrations

A THERMO FINNIGAN TSQ QUANTUM ACCESS LC/MS instrument operated in the positive ion ESI mode was used in the quantification of the parent drug and its metabolites in plasma, urine and faeces. Chromatographic separation of the extracted plasma, urine and faeces sample was performed using a THERMO HYPERSIL C₁₈ column (10 x 2.1 mm I.D., 5 µm particle size). The LC mobile phase consisted of acetonitrile : 0.1% formic acid, was run as a gradient and the total run time was 10

minutes. Quantification was performed by selected ion monitoring (SIM) or selected reaction monitoring (SRM) of the protonated precursor ion and the related product ion for etoricoxib and its metabolites using the internal standard method with peak area ratios and a weighing factor of 1/x.

Authentic standard of etoricoxib (M+H) ion at m/z 359 was available to support the characterization of parent etoricoxib in the blood, urine and faeces samples. The collision cell of the mass spectrometer was set to scan for the possible hydroxylated metabolite (M+16), carboxylation (M+32), oxidation (M+30) and glucuronic acid metabolite (M+176) of etoricoxib.

2.11. Assay procedure for celecoxib

Braeutigam et al. (2001) previously reported a solid phase extraction method coupled with liquid chromatography tandem mass spectrometry for the detection of celecoxib in human plasma. Similarly, Paulson et al., (2000a) had earlier studied the metabolism and excretion of ¹⁴C celecoxib in plasma, urine and faeces of healthy male volunteers. These two methods were employed in the study on celecoxib.

2.12. Preparation of stock solution of celecoxib and internal standard

The stock solution was prepared by dissolving 10 mg of celecoxib in acetonitrile into a 10 ml volumetric flask to give a concentration of 1 mg/ml. This was stored at 4 °C. Acetonitrile was further added to the stock solution to obtain working standard concentrations of 100, 10 and 1 µg/ml. Zaleplon (1 µg/ml) was used as internal standard. Appropriate calibration standards were prepared at a concentration of 10, 20, 50, 100, 500 ng/ml in 1 ml plasma. A volume of 200 µl of internal standard was added to each test sample and calibration standard plasma sample.

2.13. Extraction of celecoxib from plasma samples

The administration and calibration plasma samples were extracted using WATERS SepPak C₁₈ cartridges (500 mg, 3 ml). Briefly, the C₁₈ cartridge was conditioned with 3 ml of methanol followed by 3 ml of 0.1 M potassium dihydrogen phosphate (KH₂PO₄, pH 6.0). A volume of 1 ml plasma was applied on to the bed of the cartridge and passed under slow vacuum. The column was rinsed sequentially with 1 ml 0.1 M KH₂PO₄, pH 6.0 : methanol (90:10) and 1 ml 1.0 M acetic acid. Following drying for 2 minutes, the drug and its metabolites were eluted with 4 ml dichloromethane. The organic solvent containing celecoxib-related materials was dried under gentle flow of nitrogen on a CALIPER TurboVap at 40 °C. The residue was reconstituted in 50 µl mobile phase.

2.14. Celecoxib extraction recovery, assay precision and accuracy

The extraction recovery of celecoxib from plasma was evaluated in triplicates at three concentrations (10, 100 and 200 ng/ml) by comparing the peak areas of celecoxib and zaleplon to the peak areas of corresponding compounds in samples prepared by spiking extracted drug free plasma with the same amount of compound.

Within-day accuracy and precision were evaluated by analysis of quality controls at concentrations of 10, 100 and 200 ng/ml (n=5 at each concentration) on the same day. The same experiment was repeated on three different days to assess the between-day accuracy and precision. Accuracy was calculated as the percentage ratio of measured concentration to nominal concentration. Precision was expressed as the coefficient of variation. The lower limit of quantification (LLOQ) of the assay was also determined during this process.

2.15. Extraction of urine and faeces for celecoxib metabolite profiling

Urine samples were pooled as '0' hour (control), 1-12, 12-24, 24-36, 36-48, 48-60, 60-72, 72-84, 84-96, 96-108, 108-120, 120-132, 132-144, 144-156 and 156-168 hours. An aliquot of 10 ml of each urine sample was adjusted to pH 5.2. Approximately, 10,000 Fishman units of β -glucuronidase was added and incubated at 37 °C in an incubator overnight.

An amount of 10 grams of faeces was weighed and to it 10 ml of distilled water added, mixed thoroughly on a magnetic stirred for 15 minutes. Then the slurry is poured into a 15 ml centrifuging tube and centrifuged at 4000 g for 15 minutes. A volume 7 ml of the top clear liquid was adjusted to pH 6.2 and enzyme- hydrolyzed at 37 °C in an incubator overnight.

The enzyme hydrolysed samples were centrifuged and to 5 ml of urine or 5 ml of faeces, a volume of 300 μ l of zaleplon (1 μ g/ml) was added as internal standard. Samples were loaded onto WATERS SepPak C₁₈ cartridge after conditioning with methanol and 0.1M KH₂PO₄ (pH 6.0). The cartridge was washed with 1 ml 0.1M KH₂PO₄ (pH 6.0): methanol (9:1) and 1 ml of 1.0 M acetic acid and dried under vacuum for 2 minutes. The cartridge was eluted with 4 ml of dichloromethane, dried and reconstituted with 50 μ l of the mobile phase.

2.16. LC-MS-MS conditions for celecoxib administration samples

The LC unit consisted of a THERMO FINNIGAN LCQ DECA XP instrument connected to a Surveyor auto-sampler and Surveyor LC pump. Chromatographic separation of the extracted plasma, urine and faeces sample was performed using a THERMO HYPERSIL C18 column (10 x 2.1 mm I.D., 5 μ m particle size). The

mobile phase consisted of acetonitrile : 0.1% formic acid in water, run in a gradient mode. The mass spectrometer was operated in the negative ion mode performing product ion scans for celecoxib and its metabolites. Celecoxib standard was available to characterize the M-H ion of celecoxib (m/z 380) in plasma, urine and faeces. Screening for the possible metabolites, namely hydroxyl, carboxylic acid and glucuronide of the carboxylic acid metabolite was also performed.

2.17. Pharmacokinetic Methods

The plasma concentration of etoricoxib and celecoxib, and actual sampling times relative to the drug dose, were employed to estimate the pharmacokinetic parameters for each treatment in each horse. The AUC_{0-t} was the area under the plasma concentration-time curve from time 0 to time of the last quantifiable concentration after a single dose, calculated using the linear trapezoidal rule.

The C_{max} was the maximum plasma concentration observed and T_{max} was the time at which C_{max} was obtained by inspection of the concentration – time data. The terminal $t_{1/2}$ was estimated from the best fit parameters of a single exponential to the log-linear portion of the plasma concentration-time curve.

2.18. *In vitro* drug metabolism using either commercial horse hepatocytes or isolated camel liver fragments

Hill (2003) previously described an *in vitro* protocol for the measurement of compound metabolic stability using liver microsomes and the method is used in the current study. Commercial horse liver microsomes were obtained bought from Xenotech USA at a concentration of 20 mg/ml of protein. In this study, all the incubations were performed in duplicates in a shaking water bath at 37°C. The experimental protocol was the following - an amount of 1.9 mg of celecoxib or 1.79 mg of etoricoxib were weighed

and dissolved in 5 ml of DMSO to obtain a final concentration of 1 mM. The incubations were carried out by the addition of 0.1 M sodium phosphate buffer (pH 7.4, 432 μ l) containing 13 μ l microsomes, 5.0 mM MgCl_2 (5 μ l) and 10 mM NADPH (50 μ l) to each sample of either etoricoxib or celecoxib previously dissolved in DMSO. Briefly, the incubation mixture components, except NADPH, were premixed, and the resulting mixture was kept at room temperature for 3 minutes. The incubations were commenced by addition of NADPH. The experiment involved incubation of liver microsomes for either 0 (control), 30, 60, 90, 120, 150 or 180 minutes with either drug and the cofactor NADPH. At exact time intervals, particular experiment vials were removed, the reactions were stopped with 100 μ l of ice cold methanol and centrifuged at 4000 g for 20 minutes. The clear aqueous layer was dried under a stream of nitrogen and subjected to LC/MS analysis as previously described in section 2.11 and 2.19 of the method described above.

2.19. Metabolic study using fresh camel liver

Fresh camel liver was obtained from the local abattoir immediately after humanely killing the animal for food consumption. They were placed onto an ice cold saline solution and transported to the laboratory. The liver tissues were cut into small fragments of 5 mm thickness and incubated with either celecoxib or etoricoxib as described in section 2.18.

2.20. Statistical analysis of data

All pharmacokinetic and blood-borne biochemical parameters were presented according to descriptive statistics as either mean \pm standard deviation (mean \pm SD) or as mean \pm standard errors of the mean (mean \pm SEM). Where appropriate, linear regression and correlation analysis was applied to find the relationship between peak area ratios and

drug concentrations. Test (drug treated) and control (non drug treated) values were compared using Student's t-test and/ or ANOVA test. A value of $p < 0.05$ was taken as significant.

CHAPTER-3

ANIMAL CHARACTERISTICS, HAEMATOLOGICAL AND BIOCHEMICAL FACTORS IN BLOOD FOLLOWING ORAL ADMINISTRATION OF EITHER ETORICOXIB OR CELECOXIB

3.1. INTRODUCTION

Both celecoxib and etoricoxib belong to coxib class of non steroidal anti-inflammatory drugs (NSAIDs) used in the treatment of pain in osteoarthritis in humans (Bingham *et al.*, 2007). These two COX-2 NSAID drugs have the potential role to reduce pain and inflammation together with reduced gastrointestinal risks when compared to other pharmacologically-related drugs. Changes in the blood borne parameters following oral administration of such common NSAIDs like phenylbutazone and flunixin have been demonstrated previously in horses (Ansay, 1983).

To date, no proper studies have been done in large animals such as horses measuring blood-borne clinical parameters as well as any possible development of stomach ulcers that may occur in the animals following oral administration of either etoricoxib or celecoxib employing physiological doses.. Therefore, the first part of the study was designed to investigate the effects of the two COX-2 inhibitors, etoricoxib and celecoxib examining the stomach for ulcer formation and measuring the levels of a number of blood-borne biochemical parameters including RBC, WBC, platelets, MCV, MCH, haemoglobin, albumin, lipids, liver and kidney function enzymes, ions (chloride, phosphate, Na^+ , K^+ , Mg^+ , Ca^{2+} , Zn^{2+} , $^+$ and Cu^{2+}) and anions (Cl^- and phosphate) following oral administration of each coxib to the six retired race horses employing 2 mg/kg b wt of either etoricoxib or celecoxib.

3.2 METHODS

As described in Chapter 2.

3.3. RESULTS

3.3.1 Animal characteristics:

This study employed 6 healthy retired race horses (2 normal males, 2 castrated males and 2 female). The mean (\pm SEM) weight of the 6 horses was 474.1 ± 21.7 kg. The results show that within the 3 - 4 years of the study, the weight of each horse remained more or less the same. From eye observations and reports from the local Veterinary Surgeon, all the horses were of good health and they show no symptoms or signs of diseases or distress. The ages vary from 12 - 18 years (15.1 ± 2.6 years; $n = 6$). Moreover, the temperature of each horse was measured on a regular basis (either daily or weekly) and it was found to be $37.0 \pm 1.3^\circ\text{C}$). Endoscopic examination of the stomach of each horse either before or 15 days after continuous oral administration of either celecoxib or etoricoxib show that there was no significant or detectable change in the mucosa of the stomach wall (Figure 3.1 b & d, treated) compared to untreated horses (Figure 3.1 a & c). Further figure 3.2., shows the development of deep ulcers after continuous drug administration to a race horse.

3.4. Measurement of haematological and biochemical parameters

Blood samples were collected from each horse prior to administration of either 2 mg/kg etoricoxib or 2 mg/kg celecoxib and after the administration of each drug over a period of 120 hours or 5 days. These two doses were selected for treatment based on the previous studies in humans (Agrawal et al., 2003a; Paulson et al., 2001). Initial study showed no marked differences in the measured haematological and biochemical blood - borne parameters with either 2 mg/kg etoricoxib or 2 mg/kg celecoxib over the study period. Thus, all the samples were analyzed and the data pooled and presented below for both drugs.

Figure 3.3 shows the effect of oral administration of etoricoxib and celecoxib on the concentrations of different blood parameters including red blood corpuscles (RBC), packed cell volume (PCV), eosinophils (EOS), monocytes (MONO), haemoglobin (Hb), and basophils (BASO). The results show that both NSAIDs had no significant effect on these haematological parameters compared to untreated condition ($p>0.05$).

Figure 3.4 shows the effect of oral administration of etoricoxib and celecoxib on the concentrations of such blood borne parameters as total white blood cell (WBC), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), lymphocytes (LYM), mean cell volume (MCV), neutrophils (NEU) and platelets (PLT). Like figure 3.2., the data show no significant ($p>0.05$) change in the different blood parameters comparing untreated (no drug) condition with treated (drug) condition in the presence of the NSAIDs.

Figure 3.5 shows the effect of oral administration of etoricoxib and celecoxib on such blood borne biochemical parameters as creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), creatinine (CREA), blood urea nitrogen (BUN), total blood plasma protein (TP) and albumin (ALB). Again, the results clearly show that the oral administration of the two NSAID drugs had no significant ($p>0.05$) effect on these measured blood-borne markers and mediators comparing the values obtained prior to administration of the drug to the horses except for creatinine. The data showed a small, but a significant ($p<0.05$) increase in blood creatinine concentration following administration of the two NSAIDs compared to untreated conditions.

Figure 3.6 shows the effect of oral administration of etoricoxib and celecoxib on blood plasma levels of either calcium (Ca^{2+}), phosphate (Phos), sodium (Na^+), potassium (K^+),

chloride (Cl^-), copper (Cu^{2+}), zinc (Zn^{2+}) and serum amyloid a (SAA). The results clearly show oral administration of etoricoxib and celecoxib had no significant ($p > 0.05$) changes on the concentrations of the different cations and anions as well as SAA measured in plasma compared to values obtained prior and after administration of the two NSAIDs.

Since the pooled data showed a significant increase in blood creatinine levels following the administration of the two coxib drugs, it was decided to show the whole time course data for the two NSAIDs. Figure 3.7A shows the time course changes in the plasma levels of creatinine prior to (0 hour) and following administration (1 – 120 hours) of either 2 mg/kg etoricoxib or 2 mg/kg celecoxib. Figure 3.7B shows the combined data after pooling all the time point results for pre and post administration of the two NSAIDs. These results clearly show that etoricoxib can elicit a significant ($p > 0.05$) increase in plasma creatinine levels, especially between 1 and 12 hours following oral administration, compared to control (before administration of the drug). Following the time, plasma creatinine levels decrease to almost normal levels seen in pre-administration samples after 12 hours following administration. In contrast, celecoxib had little or no effect on the plasma creatinine levels compared to untreated controls (Figure 3.7 A/B).

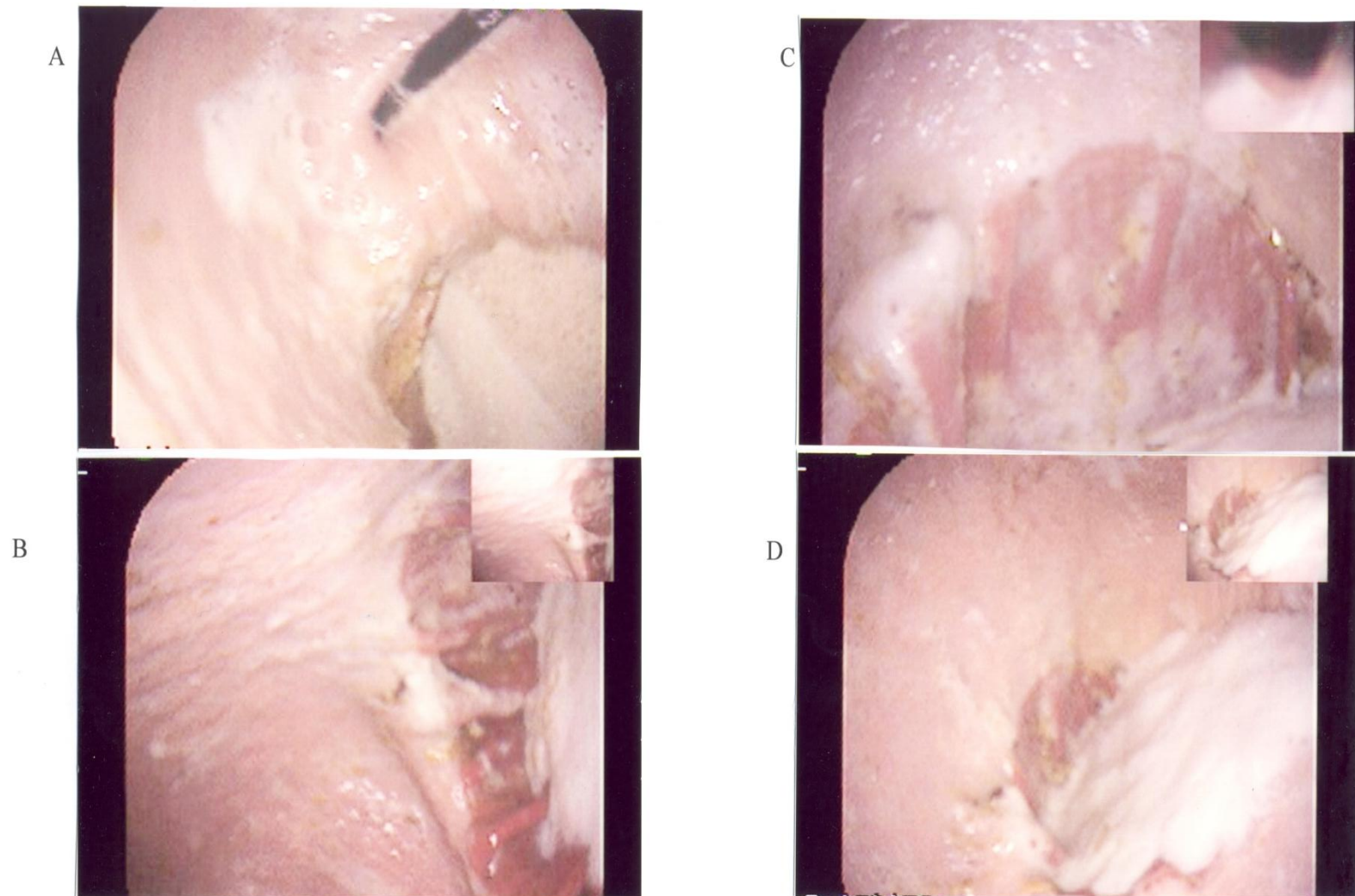


Fig.3.1: Original endoscopic photographs showing the mucosa of the stomach of untreated horses prior to administration of either 2 mg/kg b wt etoricoxib (A) or 2 mg/kg b wt celecoxib (B) and following treatment for 15 days with either etoricoxib (C) or celecoxib (D). These photographs are typical of several different experiments on the 6 horses. Note that photographs taken at days 5 and 10 show no change in the stomach mucosa.

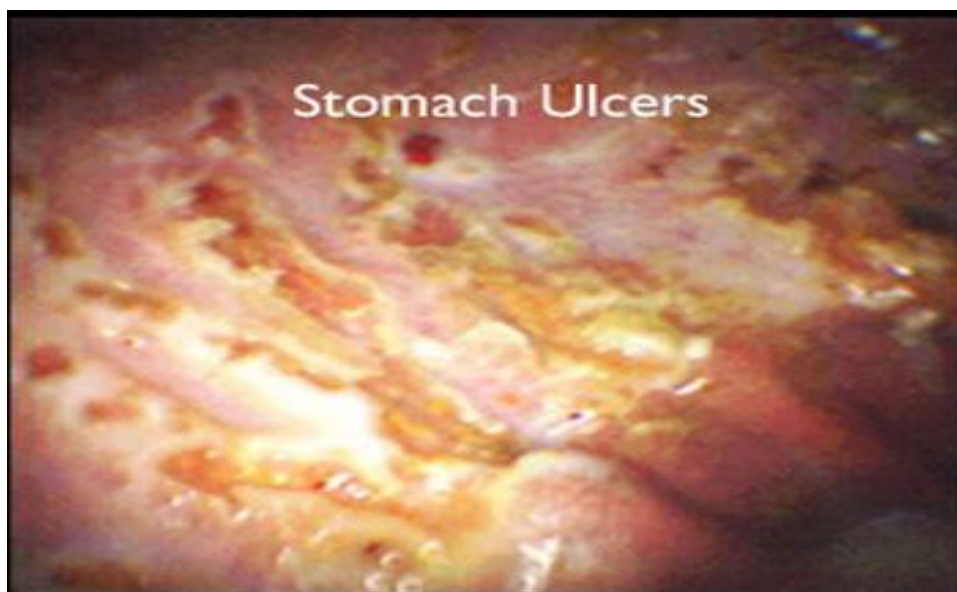


Fig.3.2. Extensive deep ulceration in a race horse after continuous medication. (taken from www.egus.com)

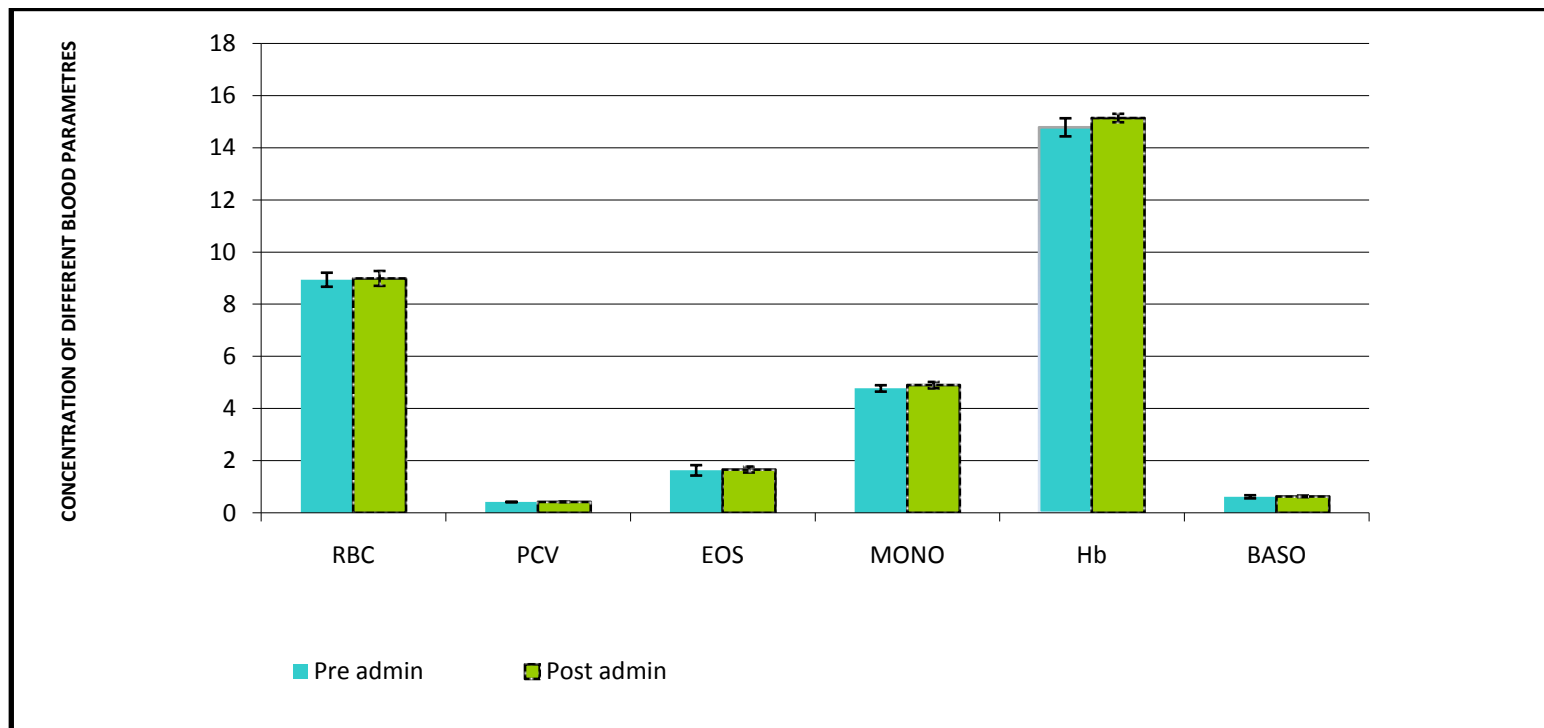


Figure 3.3: Bar charts showing the blood concentrations of RBC ($10^{12}/L$), PCV (L/L), EOS (%), MONO (%), Hb (G/dL), BASO (%) in pre admin and post administration of etoricoxib (2 mg/kg b wt) and celecoxib (2 mg/kg b wt) in horses. Data are mean \pm SEM, n =12 (combined data). Note that $p > 0.05$ for administration of the drugs compared to untreated pre-administration condition.

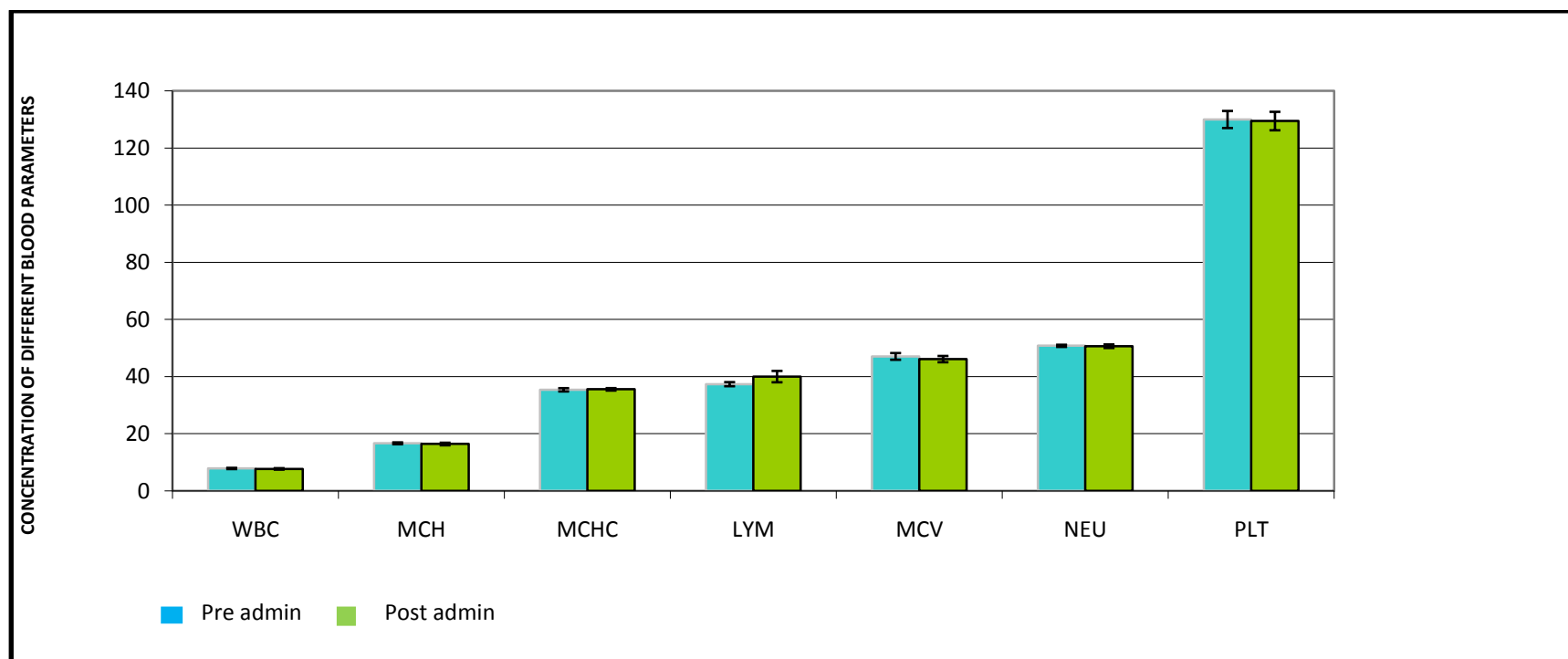


Figure 3.4: Bar chart showing the plasma concentrations of WBC ($10^9/L$), MCH (pg), MCHC (g/dL), LYM (%), MCV (fL), NEU (%), PLT ($10^9/L$) in pre admin and post administration of etoricoxib (2 mg/kg b wt) and celecoxib (2 mg/kg b wt) in horses. Data are mean \pm SEM, n =12 (combined data). Note that $p > 0.05$ for administration of the two NSAIDs compared to non-treated/pre-administration condition.

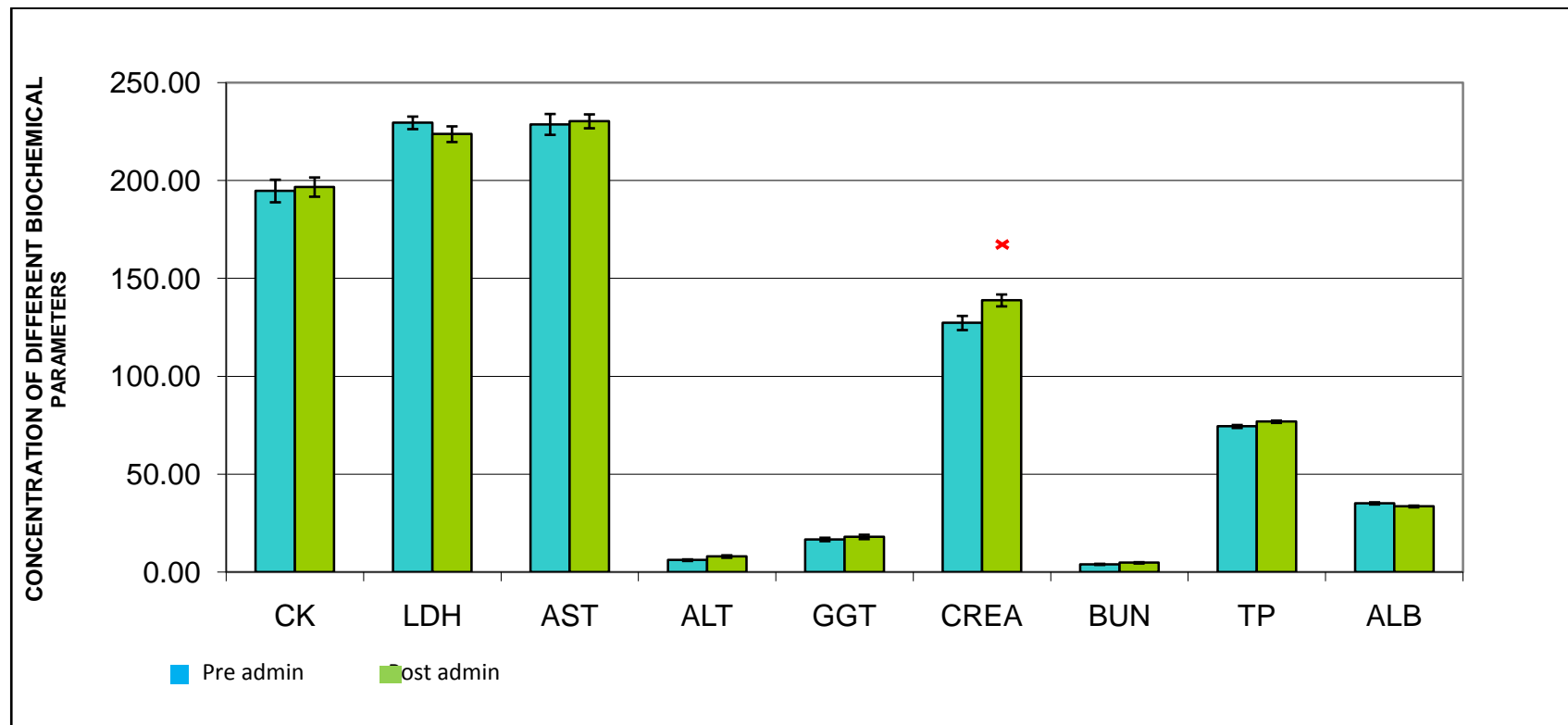


Figure 3.5: Bar charts showing the plasma concentrations of CK (U/L), LDH (U/L), AST (U/L), ALT (U/L), GGT (U/L), CREA ($\mu\text{mol/l}$), BUN (mmol/l), TP (g/l) and ALB (g/l) in pre administration and post administration of etoricoxib (2 mg/kg b wt) and celecoxib (2 mg/kg b wt) in 6 horses. Data are mean \pm SEM, n =12 (combined data). Note that $p > 0.05$ for the oral administration of the two NSAID drugs compared to untreated conditions except for creatinine where $p < 0.05$.

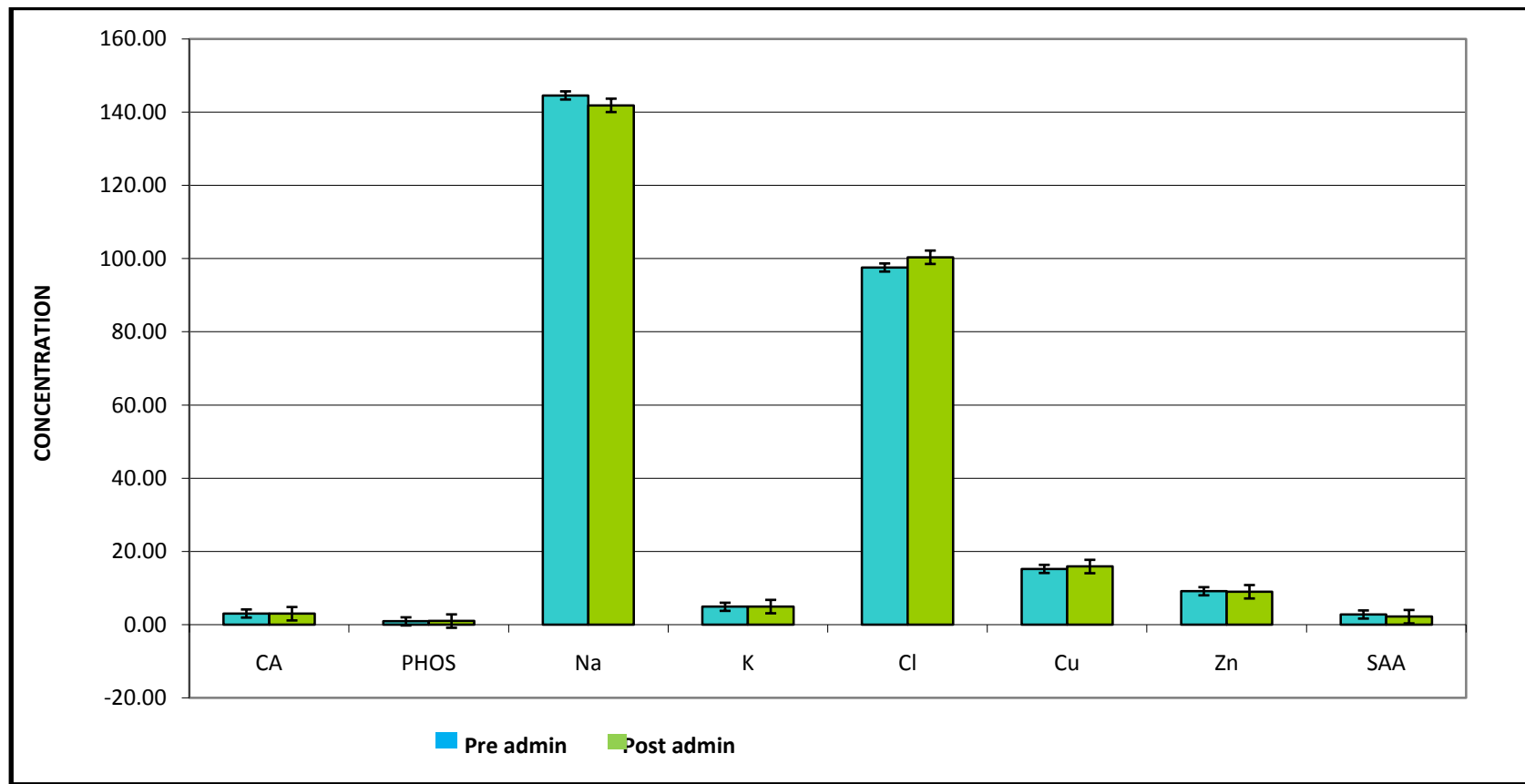


Figure 3.6: Bar charts showing the plasma sample concentrations of Ca^{2+} (mmol/l), PHOS (mmol/l), Na^+ (mmol/l), K^+ (mmol/l), Cl^- (mmol/l), Cu^{2+} ($\mu\text{mol/l}$), Zn^{2+} ($\mu\text{mol/l}$) and SAA ($\mu\text{g/ml}$), in pre administration and post administration of 2 mg/kg b wt etoricoxib and 2 mg/kg b wt celecoxib in 6 horses. Data are mean \pm SEM, n =12 (combined data), $p > 0.05$ for post administration compared to untreated or pre-administration data.

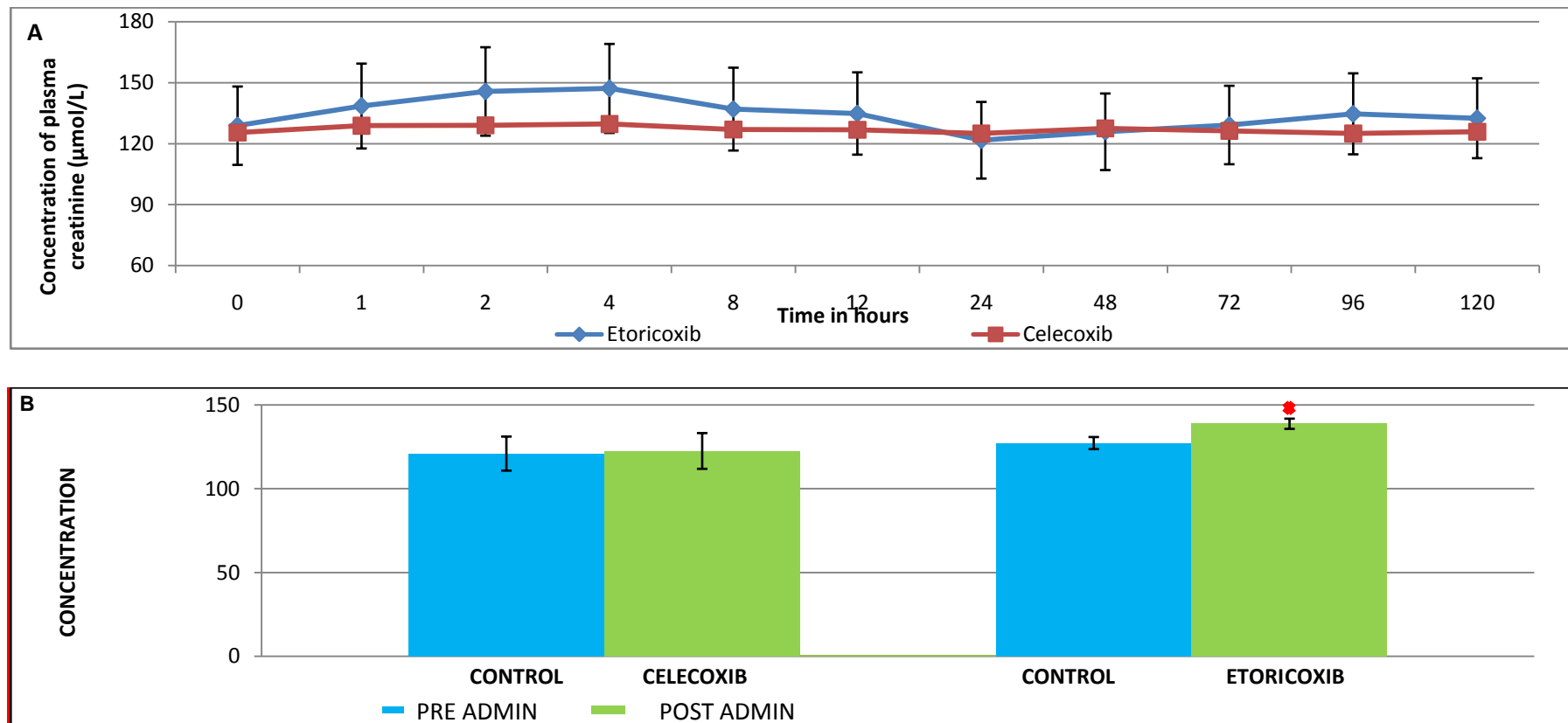


Figure 3.7: (.A) Time course changes in the plasma levels of creatinine either before (0 hour) or after the oral administration of either 2 mg/kg b wt etoricoxib or 2 mg/kg b wt celecoxib in horses. B. Bar charts showing the plasma levels of creatinine either before or after administration of either celecoxib or etoricoxib. In figure 3.6B all the data for the different time points were combined for each NSAID. Data in (A) and (B) are mean \pm SEM, n=6 ★ $p < 0.05$ for post administration compared to pre-administration of etoricoxib but not celecoxib.

3.5. Discussion

This study was designed to investigate the effects of oral administration of the two COX-2 inhibitors, etoricoxib and celecoxib in horses, measuring their adverse effects, if any, on the well being of the animals, ulcer development in the stomach and on a number blood borne haematological and biochemical parameters including whole blood, plasma enzymes and ions. NSAIDs are the most widely prescribed class of anti-inflammatory drugs to treat pain and fever (Meek *et al.*, 2010). The major pharmacological effect of the NSAIDs is to inhibit the activity of COX enzymes resulting in the decreased levels of pro-inflammatory prostaglandins. One major side effect of NSAIDs is the formation of ulcers in the stomach (Thieffin, 2005). There are different types of NSAIDs including the common aspirin and aspirin-like drugs which exert their effects by inhibiting the COX enzyme production, but in doing so they also inhibit COX-1 enzyme frequently called the house keeping enzyme giving protection to the stomach mucosa. Many new class of NSAIDs are now being developed that spare the COX-1 enzyme, thus protecting the stomach lining from formation of ulcers and bleeding (Dannhardt, 2002).

This study has employed six retired race horses of ages between 12-18 years and weighing around 474.1 ± 21.7 kg. The horses were administered with the NSAID orally and subsequently, blood samples were collected either before oral administration of the drug or after the administration of the drug over a period of 120 hours or 5 days. The results of this study have clearly shown that either NSAID drug had no significant effect on either the weights of the horses or the well being over the experimental period. Moreover, the endoscopic data also show that either drug had any detectable effect on

the mucosal lining of the stomach following continuous administration of the drug for 15 days.

The two physiological concentrations of the drugs (2 mg/kg of etoricoxib and 2 mg/kg of celecoxib employed in this study) have previously been used in other studies using the equivalent dose to the weight of the animal employing either rats (Behal *et al.*, 2009) or humans (Schwartz *et al.*, 2007). In these previous studies, it was demonstrated that administration of either celecoxib or etoricoxib had no detectable effect on the stomach lining (mucosa of the stomach). These doses (concentration vs animal weight) are considered to be safe in inhibiting COX and in reducing prostaglandin levels in the animals and at the same time they can reduce fever and inflammation (Modi *et al.*, 2012).

The results presented in this study have also shown that either 2 mg/kg etoricoxib or 2 mg/kg celecoxib had no detectable effect either on haematological and blood-borne biochemical parameters compared to untreated conditions, except for creatinine. The data presented in this study have shown that RBC and different types of WBC remained more or less the same prior to the administration of each NSAID or over a period of 120 hours following the administration of either of the NSAID. In addition, haemoglobin content, the number of platelets, blood urea nitrogen, albumin, total protein, creatine kinase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphate, serum amyloid A and gamma-glutamyl transpeptidase levels remain the same comparing values obtained before administration of each drug and post administration of the drug. Similarly, the plasma concentration of some anions like phosphate and chloride and cations as sodium, potassium, calcium, magnesium, zinc and copper remain more or less the same comparing pre-administration with post

administration. These results are more or less similar to those obtained by other studies employing either human or different animal species including rats (Behal *et al.*, 2009).

The present study has clearly shown that etoricoxib, but not celecoxib can elevate plasma creatinine levels compared to untreated (non drug) samples. This increase was more marked, 1-12 hours following etoricoxib administration. This increased level is within the reference range (88-177 $\mu\text{mol/L}$) but more at the upper level (Zapata *et al.*, 2005). This is an interesting finding since only etoricoxib can elevate plasma creatinine and not celecoxib. The reason for this difference is not known.

Plasma creatinine play a major role in determining kidney function test (Morris *et al.*, 1982). A high level denotes a tendency for kidney damage compared to normal values (Morgan *et al.*, 1977), but this value was not pathological. However, it suggests that care has to be taken when administering a high dose of etoricoxib to horses. The data also suggest that the animals can probably tolerate celecoxib much better than etoricoxib, since 2 mg/kg bwt of celecoxib had no effect on blood creatinine levels compared to untreated condition. In this study only 2 mg/kg b wt of etoricoxib was used for the oral administration. It may be worthwhile to ascertain whether a high dose of etoricoxib can induce larger increase in plasma creatinine.

Apart from the small, but significant increase in plasma creatinine following the oral administration of etoricoxib, compared to untreated condition, the present results show that the two NSAIDs are safe to be used in horses at least in the concentrations employed in the study. Moreover, they do not seem to have any adverse effects on either the stomach mucosa or the blood and its different components, including haemoglobin, liver and kidney functions, gastrointestinal tract, muscles and cation and anion imbalance in the body. In a previous study employing rats, it was demonstrated

that etoricoxib at both low anti inflammatory therapeutic (0.64 mg/kg b wt) and high pharmacological doses (1-3 mg/kg b wt) help to replenish protective cells in the colon via changes in nitric oxide (NO) metabolism. Moreover, these two doses did not interfere with both the growth and the health of the animals as well as functional parameters of the kidneys and colon (Behal *et al.*, 2009).

In other studies, it was demonstrated that chronic administration doses (10 mg/kg b wt and 30 mg/kg b wt) of etoricoxib to rats can produce marked adverse effects on blood pressure and other haematological parameters. The significant elevation in mean arterial blood pressure was associated with concurrent increases in RBC, platelets, haematocrit and haemoglobin (Baracho *et al.*, 2009; Tacconelli *et al.*, 2004). Together, the results obtained in the present study and those from the previous studies have clearly shed light on the correct anti inflammatory therapeutic dose of the NSAID when administering them to animals, including humans and horses.

Although nonsteroidal anti-inflammatory drugs are widely used to control pain because of their efficacy and safety profile, there are concerns about their safety on long term usage. A vast majority of healthy human subjects have been found to tolerate the drug without adverse effects while, a minor percentage of individuals are susceptible to serious renal toxicity (Bennett *et al.*, 1996). The liver is the major organ for metabolism and the kidney plays a major part in the concentration and elimination of xenobiotics and is more susceptible to their toxic effects. These adverse events are manifested by elevation serum creatinine and blood urea nitrogen leading to permanent damage of kidney tissues in persons who have abused analgesic mixtures for many years (Dunn, 1984). All NSAIDs inhibit cyclooxygenase, the enzyme that is required for the conversion of arachidonic acid into prostaglandins. These prostaglandins are involved in

the inflammatory process and are also present in the kidneys where they balance the effects of vasoconstrictions by causing vasodilatation leading to renal blood supply and glomerular filtration. In case of renal toxicity due to NSAIDs, the renal blood flow is decreased, which results in decreased glomerular filtration.

In normal steady-state conditions, the daily urinary creatinine excretion is equal to daily creatinine production. In situations of renal toxicity due to NSAIDs, the glomerular filtration rate falls, with subsequent diminished excretion of creatinine resulting in a rise in serum creatinine concentration (Perrone *et al.*, 1992). An elevation of serum creatinine occurs within 24 hours of exposure to the NSAID and falls back to normal levels after complete excretion or discontinuation of the drug (Garella & Matarese, 1984). Several drugs such as salicylates, cimetidine, corticosteroids and vitamin D metabolites have been reported to increase plasma creatinine levels without influencing its glomerular filtration (d'Agate, 1996; Baylis *et.al.*, 1979; Compston *et al.*, 1979; Dubb *et al.*, 1978).

The incidence of NSAID-induced renal complications are relatively low and are typically fully reversible if the offending NSAID is discontinued. The cardinal signs of NSAID-associated nephrotoxicity are an elevated serum creatinine and blood urea nitrogen with diminished urine volume and a swift and timely discontinuation will reverse the condition to normal levels, if not the syndrome may progress rapidly, necessitating dialysis (Blackshear *et al.*, 1985). The selective COX-2 inhibitors are a unique and relatively new class of NSAID, commonly prescribed for pain relief mostly for symptoms of rheumatoid and osteoarthritis in patients with gastritis and peptic ulcers. Most trials, comparing selective COX-2 inhibitors with traditional NSAIDs,

emphasis has been directed to the gastrointestinal safety and the renal safety profile study is generally excluded or not studied (Rifkin & Perazella, 2005).

Brater *et al.*, (1985) studied the effect of ibuprofen, naproxen and sulindac in men and concluded that these traditional NSAIDs did not have any altered renal effects at doses of 600 mg, 375 mg and 200 mg, respectively on observing the values of blood urea nitrogen and serum creatinine in eleven healthy human volunteers. Similarly, Whelton *et al.*, (1990) studied the renal effects of ibuprofen, at doses of 800 mg three times daily; piroxicam, 20 mg daily; and sulindac, 200 mg twice daily for 11 days in patients with asymptomatic renal failure. Serum creatinine, effective renal plasma flow, glomerular filtration rate, systemic drug kinetics and peripheral blood platelet thromboxane B₂ production were monitored. They concluded that ibuprofen; the widely used nonprescription drug increased the serum creatinine level from day 2, which may lead to acute renal failure, thus, leading to discontinuation of the trial. Thereafter, serum creatinine levels decreased and approached to baseline levels. Moreover, no statistically significant changes in serum creatinine, glomerular filtration rate were observed in piroxicam and sulindac administration.

More recently, acetaminophen has been found in some patients to develop asymptomatic aminotransferase elevation with more than five days of treatment. Incidentally, no published prospective studies of liver injury has been reported (Watkins *et al.*, 2006). Dixit *et al.*, (2010) reported that 15 patients studied at The University Medical Center, Tucson, USA, had abnormal serum creatinine levels after ingesting recommended dose of NSAIDs and took 37 ± 42 days for the normalization of serum creatinine levels after discontinuation of the administration.

Several case reports of renal toxicity in adolescents using NSAID have been reported by Nakahura *et al.*, (1998). These are very particular with the use of ibuprofen intermittently for 9 months, in another case the use of ibuprofen daily for 6 months and in the third case, the use of a combination of naproxen for a week, followed by ibuprofen for several weeks and ketorolac intermittently for 4 months. All these case reports were asymptomatic for a week and later developed an increase in serum creatinine leading to chronic interstitial fibrosis of the kidney. The nephrotoxicity was resolved within 2 months with the withdrawal of these non-salicylated NSAID.

Sturmer *et al.*, (2001) in their study on 802 patients undergoing joint replacement therapy estimated renal function using serum creatinine levels and suggest that users of NSAIDs with a short half-life (less than 4 hours) were not more likely to have impaired renal function, whereas use of NSAIDs with longer half-life (4 hours or longer) are more prone to impaired renal function.

Hegazy *et al.*, (2011) studied the cardio-renal effects of the newer NSAID celecoxib in seven hundred and ninety two patients with arthritis, administering celecoxib 400 mg twice daily compared with 396 patients taking ibuprofen 300 mg three times a day. They report that celecoxib had lower incidence of hypertension and edema compared to ibuprofen. Serum creatinine was significantly increased in patients treated with ibuprofen in comparison with celecoxib. In another report with dipyrrone, it was found to cause acute renal insufficiency in children with increased levels of urea, creatinine and other electrolytes within 1 week of drug exposure and normalization of serum urea and creatinine was achieved within 3-16 days after discontinuation (Abu-Kishk *et al.*, 2010).

Zhao *et al.*,(2001) compared the renal related adverse reactions of rofecoxib and celecoxib based on adverse drug reactions in the World health Organization/Uppsala Monitoring Centre and report that as with traditional NSAIDs, both rofecoxib and celecoxib were associated with renal related adverse drug reactions. However, rofecoxib had showed a significant increase in serum creatinine (2.38 vs 0.70; $P<0.01$) compared to celecoxib resulting in withdrawal of celecoxib from the Australian market to adverse renal effects.

In a controlled arthritis trial with 5000 patients over a two year period, celecoxib showed no increase in both serum creatinine concentration and blood urea nitrogen. Thus, it was concluded that celecoxib is well tolerated by patients having a risk for NSAID-induced renal toxicity, such as the elderly and those with hypertension (Whelton, 2001).

Two large studies such as the CLASS and VIGOR has concentrated on the comparative gastrointestinal safety and renal adverse reaction of the coxibs and the non-selective NSAIDs. In the CLASS study, celecoxib (400 mg twice daily) was compared with diclofenac and ibuprofen in the treatment of osteo and rheumatoid arthritis. It has been reported that a small proportion of patients developed renal impairment as judged by an increase in serum creatinine to over 159 μM compared to an higher incidence in patients taking either diclofenac or ibuprofen (Silverstein *et al.*, 2000b). Similarly VIGOR study compared rofecoxib (50 mg) and naproxen (500 mg twice daily) and reported that adverse reactions related to renal function were not significantly different in both administration trials (Bombardier *et al.*, 2000)

Curtis *et al.*, (2004) studied the renal effects of etoricoxib (60, 90, 120 mg) versus naproxen (500 mg/ twice daily), ibuprofen (800 mg/thrice daily) and with that of placebo in about 4,700 patients between June 1999 and November 2000. These authors conclude that there was no clinically meaningful dose related incidence of elevated serum creatinine in patients receiving etoricoxib irrespective of the dose. Furthermore, they report that the risk of renal adverse events were low when compared with naproxen and ibuprofen.

Thus, in conclusion, the results of the present study have shown that low anti-inflammatory therapeutic doses of either celecoxib or etoricoxib can produce virtually no adverse effects on the six retired race horses employed in the study. Based on the present findings, it can be concluded that the two specific COX-2 inhibitors, celecoxib and etoricoxib are safe drugs to use at therapeutic doses to treat pain, at least in horses.

CHAPTER – 4

PHARMACOKINETICS AND METABOLISM OF CELECOXIB AND ETORICOXIB IN HORSES

4.1. INTRODUCTION

NSAIDs are valuable analgesic therapeutic agents that are widely prescribed worldwide for the relief of pain in spite of significant health burden due to their problematic side effects (Stevenson, 1984). Gastro-protective agents are generally used to decrease their harmful toxicity. The advent of COX-2 class of drugs offer better tolerance and efficacy compared to traditional NSAIDs (Antman *et al.*, 2007). Etoricoxib and celecoxib are two new generation COX-2 inhibitors in the market for pain relief in conditions of severe osteoarthritis (Turner *et al.*, 2004). The pharmacokinetics and metabolism of these two drugs have been well established at least in humans and to some extent in small animals such as rats and rabbits (Radwan *et al.*, 2012;Vadnerkar *et al.*, 2006), but there is limited scientific information on the therapeutic usage of these two NSAIDs in large animals like horses and camels, which are involved in the racing industry. As such, it would be meaningful to study both their pharmacokinetics and metabolism in horses. In turn, this could lead to possible detection in post race blood or urine samples. Moreover, the results may give a guideline to the racing industry, the owners of the race horses and the Veterinary Clinical Doctors of a possible withdrawal times using these human medications in horses before the race. As such, this study investigated the time course changes in the pharmacokinetics and metabolism of the two NSAIDs, celecoxib and etoricoxib for comparison,

4.2. Method

As described in chapter – 2

4.3. Results

4.3.1. Optimisation of extraction method for celecoxib and etoricoxib

The detailed method for the extraction of celecoxib and etoricoxib is described in chapter -2 of this thesis. The procedure for extraction is based on literature published by Braeutigam *et al.*, (2001) for celecoxib and by Braeutigam *et al.*, (2003) for etoricoxib. In the previous studies, most of the publications described a solid phase extraction method using a Bond Elut C₁₈ cartridge for celecoxib and a cationic exchanger cartridge such as Oasis HLB marketed by Waters, USA. The extraction method in the present study used a Bond Elut C₁₈ cartridge supplied by Agilent USA., and a cationic exchanger cartridge named IST HCX supplied by Biotage, Sweden. Isolute HCX cartridges are similar to Oasis HLB cartridges in all aspects.

The experiments conducted at the Equine Forensic Unit, CVRL, Dubai, UAE, is a sports drug testing laboratory, accredited by National Association of Testing Authorities (NATA), Australia since 1999. The laboratory analyses approximately 14,000 samples annually using procedures as described in chapter-2 for the presence of performance enhancing drugs such as NSAIDs, stimulants, β -blockers, anabolic steroids etc. These procedures are commonly used in all drug testing laboratories worldwide. Moreover, the extraction methods employed in these experiments are similar to those described by Braeutigam *et al.*, (2001 & 2003) and are accredited by NATA, Australia. Celecoxib and etoricoxib are two newly introduced NSAIDs in the market and such similar drugs such as firocoxib, rofecoxib and valdecoxib are routinely screened at this laboratory on a day to day basis. The solid phase extraction cartridges and all reagents used in the present experiments are same as published in the literature.

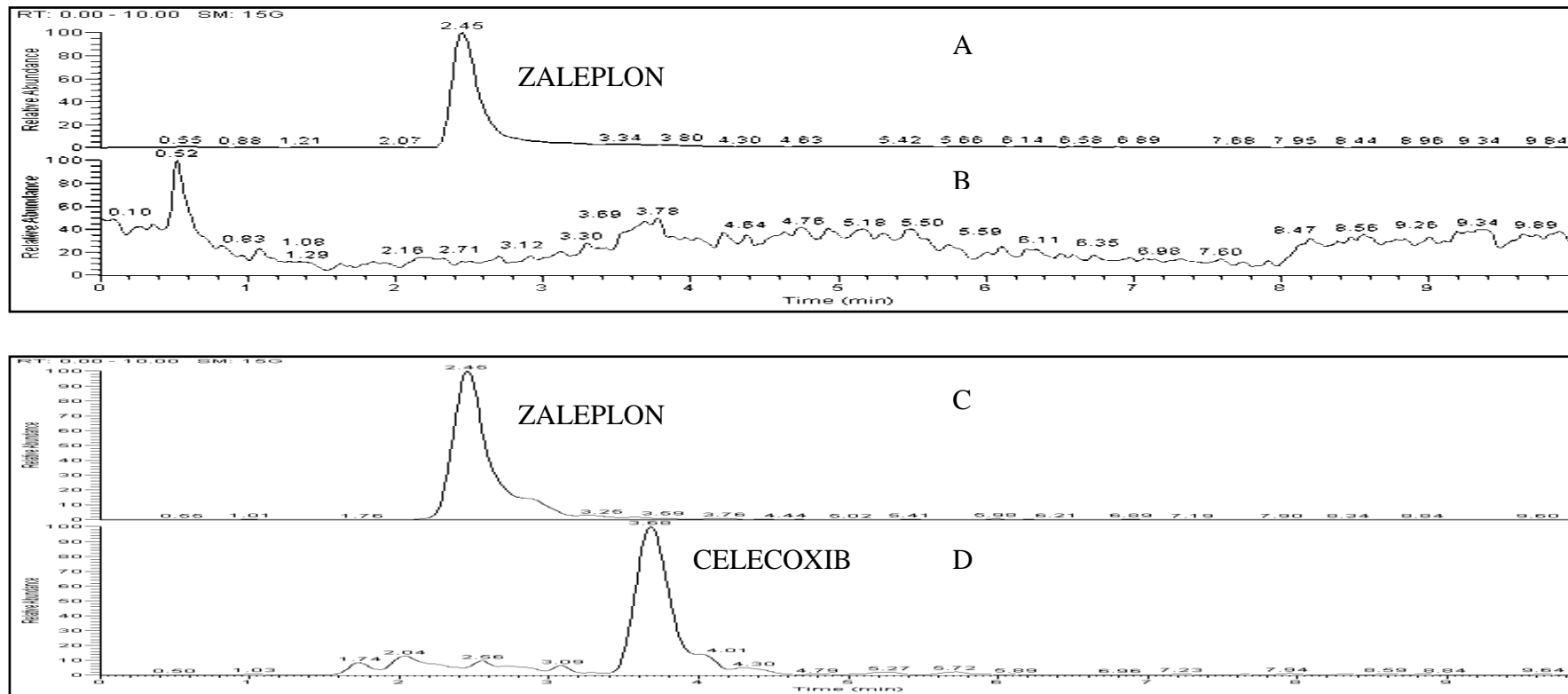


Figure 4.1: Original chart recordings of chromatograms showing (A) the retention time of zaleplon (IM), (B) the absence of any signal at the RT of celecoxib, (C) the peak for zaleplon (IS) and (D) the peak for celecoxib spiked in blank plasma. Chromatograms are typical of 6 such different experiments employing 6 different horses.

4.3.2. Celecoxib analytical method validation

Selectivity and specificity

The selectivity and sensitivity of the extraction and analytical method for celecoxib and its metabolites were investigated in plasma samples obtained from horses either before or after the administration of the drug. Figure 4.1 shows typical original chart recordings of chromatograms of (A) blank horse plasma spiked with zaleplon as internal standard, (B) blank horse plasma without any endogenous traces of celecoxib, (C) blank horse plasma spiked with zaleplon as internal standard and (D) blank horse plasma spike with the drug, celecoxib at 10 ng/ml. The results show that at an optimum flow rate of 200 μ l/min for the mobile phase and other chromatographic conditions described in chapter 2., the peaks for celecoxib and the internal standard were well resolved and interestingly, no peak tailing was noticed during the measurements.

The present results also show that no interfering peaks were observed in the chromatograms of blank horse plasma or the chromatograms of blank plasma from all 6 healthy horses participated in the study. In addition, the results show that no endogenous plasma components were eluted at the retention time of celecoxib, when extracted using C₁₈ cartridges and subjected to LC/MS/MS analysis using THERMO HYPERSIL C₁₈ analytical column.

Precision and accuracy

The intra-day and inter-day assay precision and accuracy for low, medium and high concentration of celecoxib in horse plasma are summarized in Table 4.1 showing a percentage of CV, ranging from 5.6 to 9.8 and 4.9 to 8.0 respectively. The limit of

detection for celecoxib giving a good signal to noise ratio of above 3:1 was 5 ng/ml and the limit of quantification was 10 ng/ml.

Table 4.1: Intra and inter-day variability of the LC/MS assay for determination of celecoxib in three different concentrations in plasma (n=5). Data are mean \pm SD.

C (ng/ml)	Intra-day variability		Inter-day variability	
	Mean \pm SD	CV%	Mean \pm SD	CV%
10	10.42 \pm 1.02	9.80	9.95 \pm 0.80	8.03
100	99.8 \pm 5.7	5.7	101 \pm 4.44	4.4
200	199.81 \pm 11.2	5.6	201.6 \pm 9.8	4.9

4.4. Pharmacokinetics of celecoxib

Table 4.2 shows that the pharmacokinetic parameters of celecoxib given as an oral dose and at a concentration of 2 mg/kg but using LC/MS methods. All the animals used in this study following oral administration of celecoxib were healthy and they show no sign of distress as described in chapter 3 of this study. The results show that after oral administration of celecoxib at a concentration of 2 mg/kg bwt, an average maximum concentration (C_{max}) was found to be 1,157.87 \pm 323.58 ng/ml, while the time to reach peak plasma concentration (T_{max}) was just 4.09 \pm 1.6 hrs. The extent of drug absorption which was characterized by the area under the plasma concentration-time from zero to infinity ($AUC_{0-\infty}$) was shown to be 29927.56 \pm 6751.04 ng/ml/hr. The mean (\pm SEM) plasma concentration-time profile of celecoxib after administration of celecoxib to the six healthy retired race horses is also given in Figure 4.2 for comparison. A chart showing real values recorded during the pharmacokinetic experiments for celecoxib is added to the Appendix (table 3).

Table 4.2: Calculated pharmacokinetic parameters of celecoxib following oral administration of 2 mg/kg in horses. Data are mean \pm SD, n=6.

Parameters	(Mean \pm SD)
C_{\max} (ng/ml)	1,157.87 \pm 323.58
K	0.03 \pm 0.01
t_{\max} (hours)	4.09 \pm 1.60
$t_{1/2}$ hours	25.52 \pm 8.40
AUC _{total} (ng/ml/hr)	29927.56 \pm 6751.04
$C_{(0)}$	537.46 \pm 228.13

4.5. Metabolism and excretion of celecoxib in plasma, urine and faeces

Following oral administration of 2 mg/kg body weight of celecoxib, the metabolism of the drug was determined in plasma, urine and faeces over time.

Plasma: The mean (\pm SEM) cumulative excretion of celecoxib in plasma after a single oral dose in 6 different horses is shown in Figure 4.2. The result show that there was a rapid increase in plasma levels of celecoxib reaching a maximum of about 1 μ g/ml at about 4 hours after administration. Thereafter, the level of celecoxib decreases gradually reaching detectable levels up to around 80 hours following administration. The product ion scan for either a probable hydroxylation or carboxylation of the parent drug yields traces of metabolite 1 (M1) which could not be quantified due to its low abundance seen in plasma. In contrast metabolite 2 (M2) was excreted in abundant quantities comprising of more than 95% of the metabolite produced from celecoxib.

Urine: The urine LC/MS profile has indicated that celecoxib is extensively metabolized in horse and this is indicated by its low concentration in urine samples. Figure 4.3 shows the excretion of celecoxib in (A) the six different horses employed in the study and (B) the results for the mean (\pm SEM) excretory data for all six animals. Celecoxib peaks at around 18 – 24 hrs with a maximum concentration of 4.8 ± 1.44 ng/ml (mean \pm SEM, n=6) after oral administration. The results on figure 4.3 also show that celecoxib can be detected in urine for up to 60 hours after a single dose of oral administration to each horse. This low concentration in equine urine suggests extensive metabolism and therefore a high probability of urinary metabolites.

Hydroxylation and carboxylation are the simplest metabolic reaction that occur in more than 95% of xenobiotics that enter the biological system (Leucuta *et al.*, 2006). Based on this assumption, this study further investigated the metabolites produced from celecoxib in urine samples using either product ion scan for a hydroxyl (addition of m/z 16 Da) or carboxylation (addition of 30 Da). The results yielded both M1 metabolite of m/z 396 and M2 metabolite of m/z 410. The M1 metabolite having a m/z 396 is 16 mass units higher than the parent molecule celecoxib acquired in the negative ion mode. An 'OH' group has a mass of 16 and gets attached to the methyl group of celecoxib molecule to form hydroxymethyl celecoxib. Further the hydroxymethyl celecoxib attaches oxygen to its structure to form carboxy celecoxib (COOH-celecoxib).

Figure 4.4 shows the time course of the excretion profile of M2 in the six different horses (A) and (B) shows the mean (\pm SEM) levels of celecoxib, COOH celecoxib (M2) and hydroxymethyl celecoxib (M1). In the absence of commercially available metabolite standards and quantification based on mass spectral identification, the data

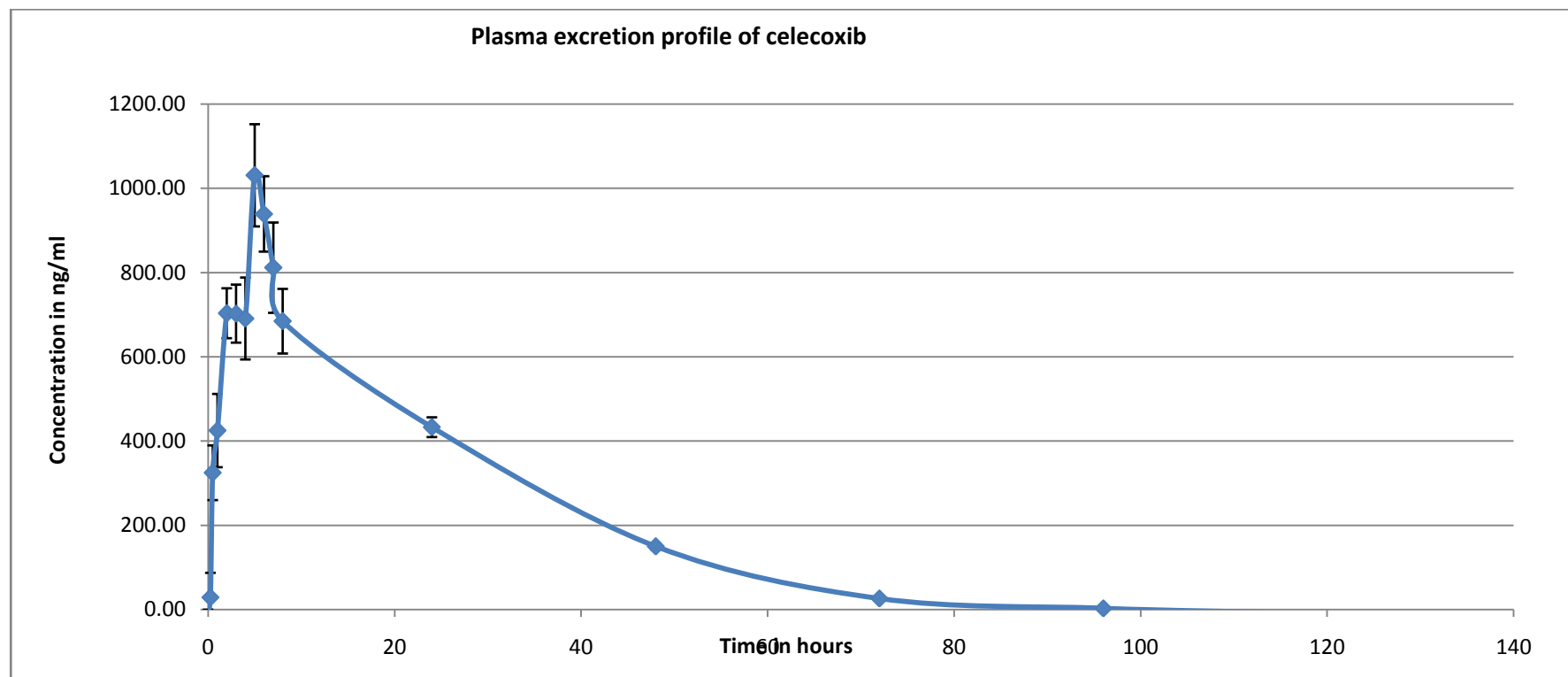


Figure 4.2: Time course changes (mean \pm S.E.M) of plasma concentration of celecoxib following oral administration of a single dose of 2 mg/kg b wt to 6 healthy horses. Note the rapid increase in plasma levels of celecoxib and its slow decline possibly due to its metabolism.

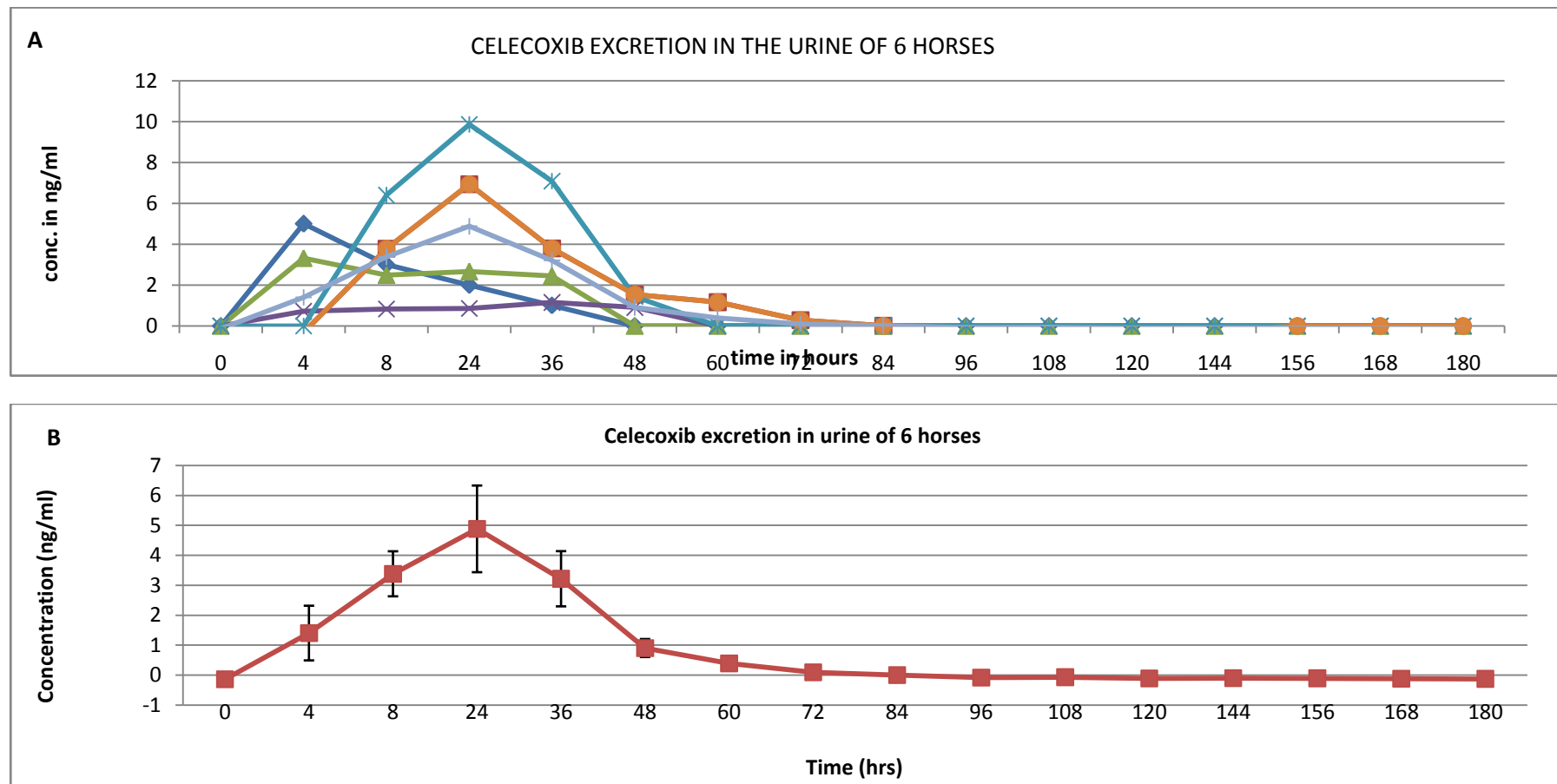


Figure 4.3: Time course changes in celecoxib excretion in (A) urine samples of 6 horses employed in this study and (B) the mean (\pm SEM) data for the six animals. Note the small detection volume (ng/ml) for celecoxib in urine.

show that about 105.53 ± 19.09 ng/ml of COOH-celecoxib and ≈ 3 ng/ml of OH-celecoxib can be measured at 24 hours after an oral dose of 2 mg/kg of the NSAID drug to the 6 horses. The COOH celecoxib metabolite of celecoxib accounts for significantly ($p < 0.05$) more than 90% of the metabolite formed and it is the major metabolite in urine followed by a small trace amounts of M1 metabolite (OH-methyl celecoxib) and the parent celecoxib, both accounting for 1-2% in urine. The high abundance of COOH celecoxib in urine suggests that celecoxib undergoes hepatic metabolism where it is converted by hydroxylation to form hydroxymethyl celecoxib, which is further oxidized at the hydroxyl group to form the major COOH celecoxib metabolite. In passing, it is noteworthy that there is no evidence for the presence of any glucuronic acid conjugate when using a neutral loss screen in the LC/MS screen.

Faeces: Figure 4.5 shows the excretion of celecoxib and its M2 metabolite (COOH-celecoxib) over a period of 5 days in faeces. The results show that at day 1 following oral administration, celecoxib is detected at a concentration of 287.77 ± 53.84 ng/ml (mean \pm SEM, $n=6$) and is seen as the major drug component compared to a significantly ($p < 0.05$) lower level of COOH celecoxib metabolite at day 1. The concentration of the COOH celecoxib metabolite was about 91.12 ± 40.22 ng/ml (mean \pm SEM, $n=6$). After 2 days following oral administration, the levels of either celecoxib or COOH-celecoxib decreased to almost zero level in the faeces samples. There were no traces for the presence of hydroxymethyl celecoxib from the day of administration till the final day of faeces collection.

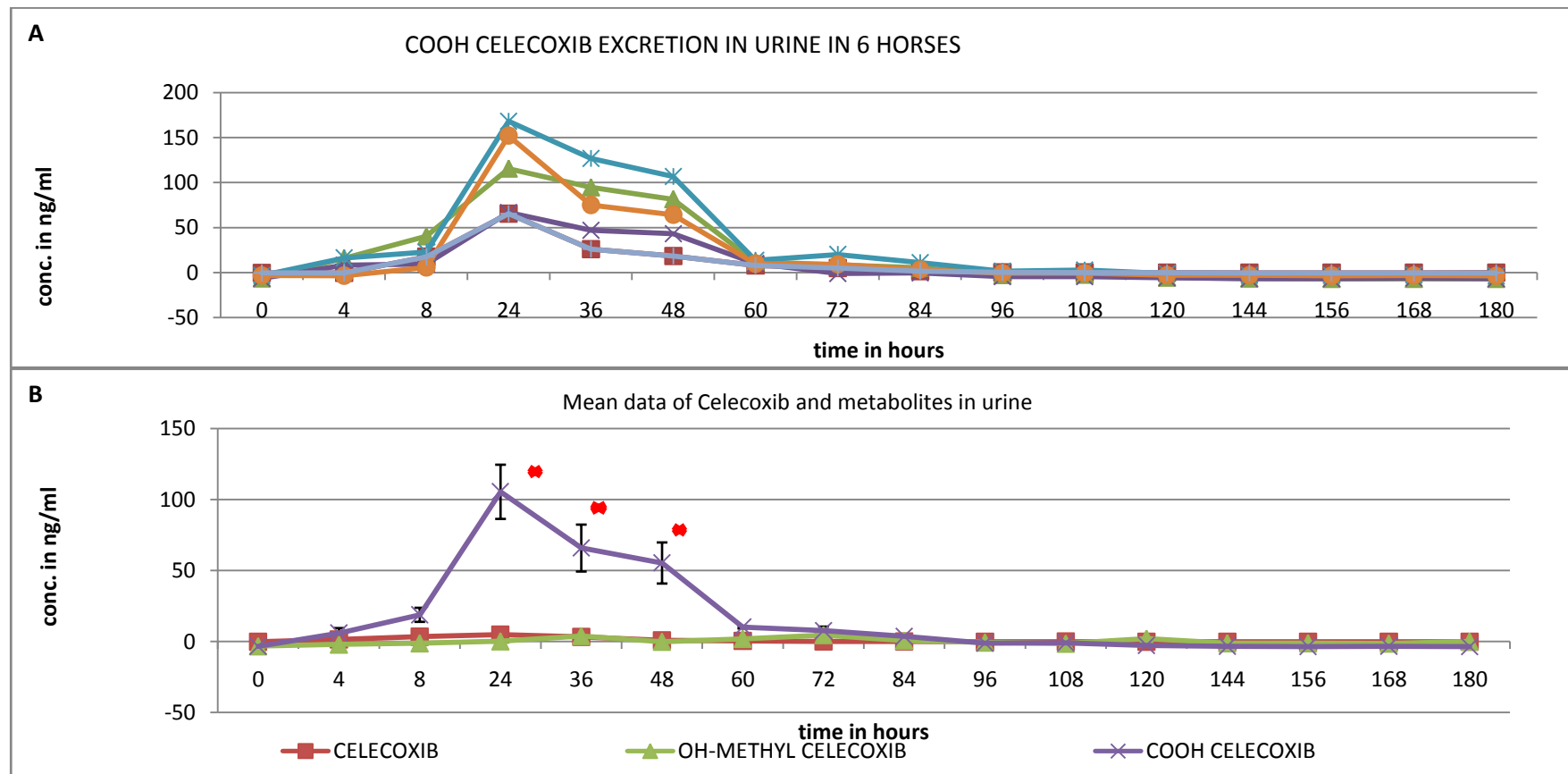


Figure 4.4: Time course changes in levels of (A) COOH-celecoxib (M2 metabolite) in 6 different horses and (B) the mean (\pm SEM) levels of celecoxib (solid squares), OH-methyl celecoxib (solid triangles) and COOH-celecoxib (asterisk) in urine samples. Note the large excretion of COOH-celecoxib (M2 metabolite) in urine compared to M1 (OH-methylcelecoxib) or celecoxib. $n=6$; * $p<0.05$ for COOH-celecoxib compared to either celecoxib or OH-methylcelecoxib.

4.6. Identification of COOH-celecoxib and OH-methyl celecoxib

The results so far have shown that celecoxib can be broken down in the body of the horses to produce two metabolites, the major COOH-metabolite (M2) and the minor OH-methyl celecoxib (M1) which are excreted predominantly in urine and faeces, but not in plasma. The next logical step of the study was to ascertain whether the two metabolites have been derived from the parent compound celecoxib in order to substantiate the findings. This is of particular significance in drug metabolism especially in the racing industry. Thus the LC/MS analysis was used to determine whether the two metabolites were true ‘finger prints’ of celecoxib. This is a novel analytical technique which is done only by LC/MS.

Figure 4.6 shows the mass spectrum of celecoxib acquired in the negative ion mode with (M-H) ion m/z 380 and which has fragmented to give products of m/z 316, 296, 276 and 256. Interpretation of the mass spectrum of m/z 316 could have arisen from the loss of SO_2 (64) from the parent molecule. The product ions m/z 296, 276 and 256 are generated by the sequential loss of HF (20) from the m/z 316. Similarly, when celecoxib was acquired in the positive ion mode, it gives a (M+H) m/z of 382 which loses a m/z of 20 (HF) sequentially to give fragments of 362, 342 and 322 (see Figure. 4.7). Blood, urine and faeces samples acquired in either negative or positive ion mode to undergo the corresponding fragmentation has well characterized sequential loss of HF.

Metabolite M2 – The (M-H) ion of M2 metabolite of m/z 410 in the negative ion mode as shown in figure 4.8, has a mass to charge unit of 30 more compared to celecoxib which has a mass of 380. This finding suggests that M2 metabolite could be a carboxylic acid metabolite of celecoxib which could be detected up to 96 and 48 hours in urine and faecal samples, respectively following oral administration. The fragmentation or the collision induced dissociation of m/z 410 produces characteristic

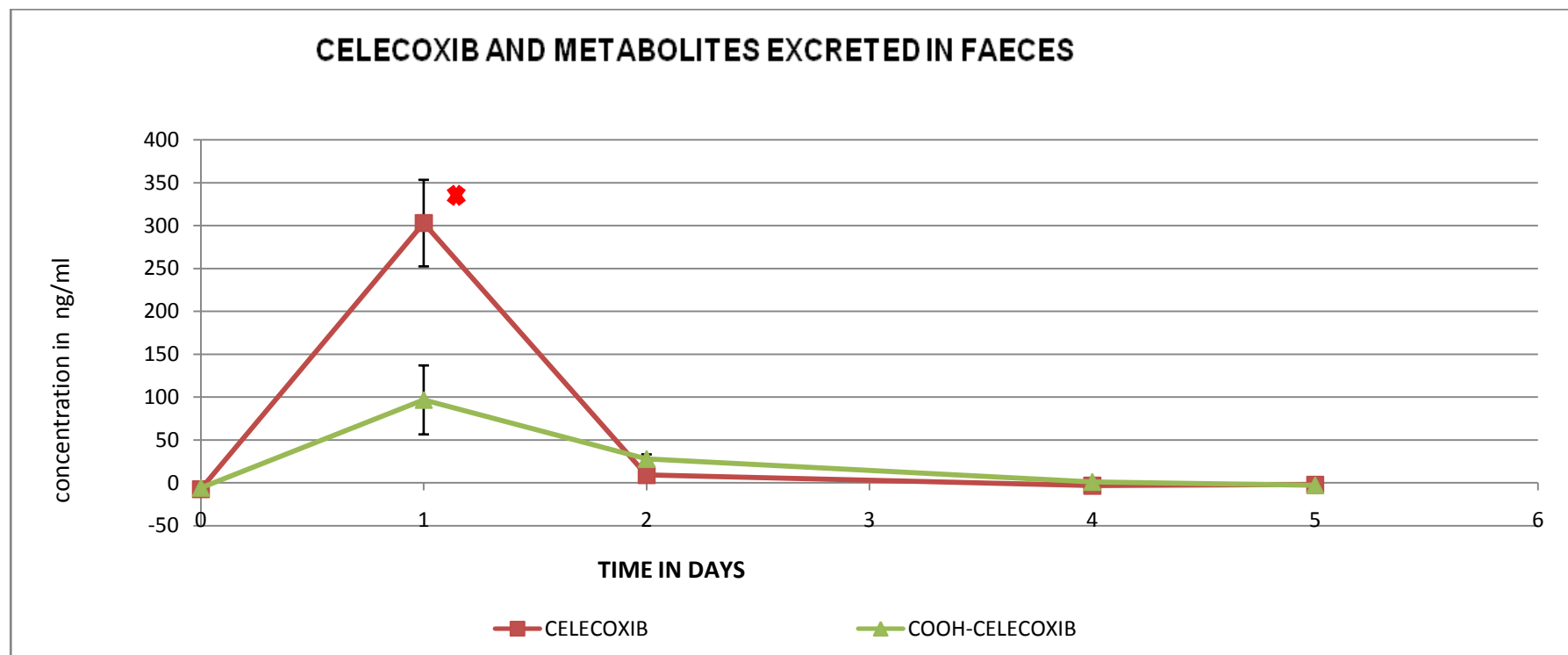


Figure 4.5: Time course changes of faecal excretion of celecoxib (solid squares) and its M2 metabolite COOH-celecoxib. Data are mean \pm SEM. $p < 0.05$ for celecoxib compared to COOH celecoxib. Note that maximal excretion of parent drug and M2 metabolite occurred at day 1 following oral administration.

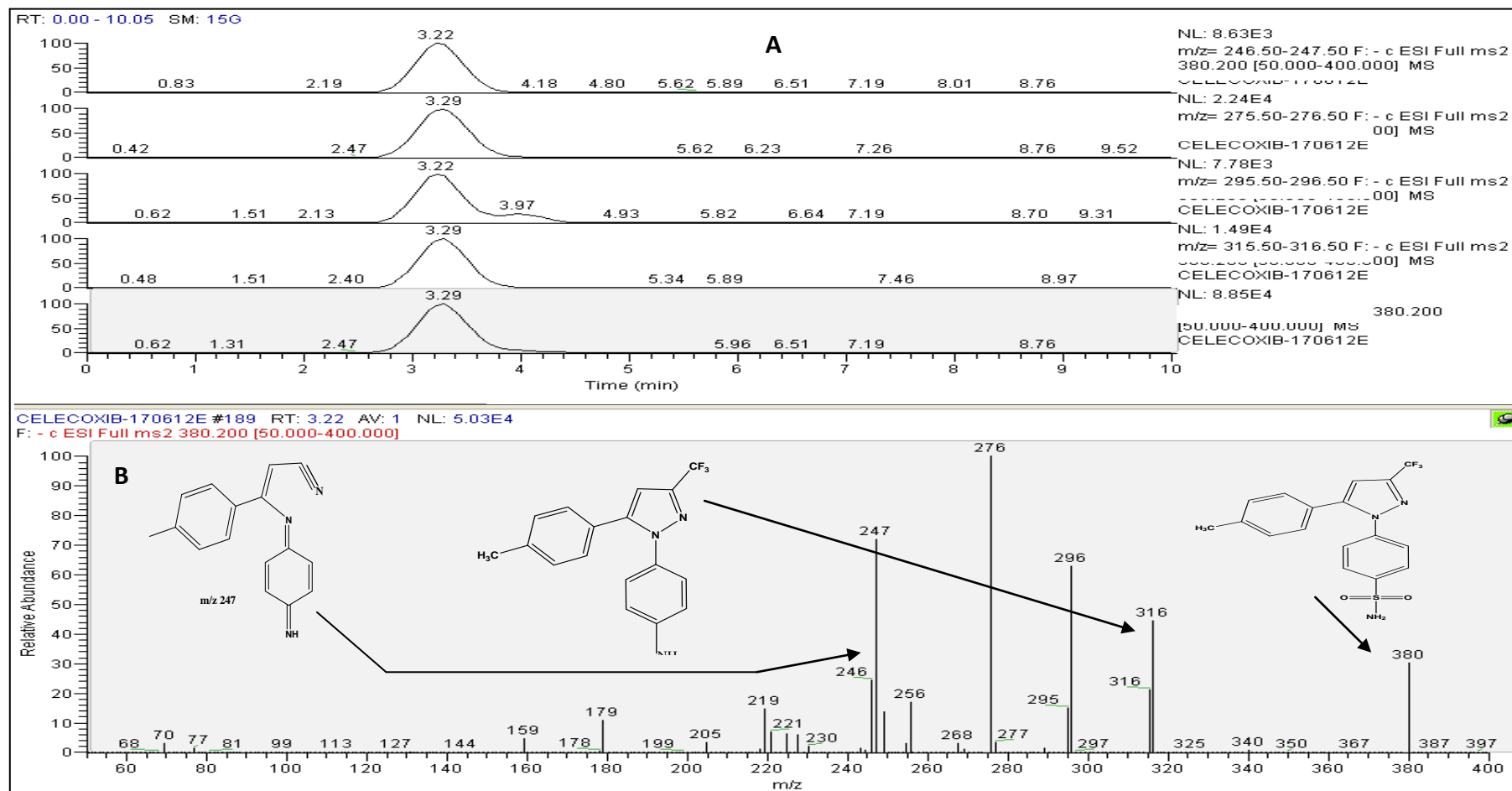


Figure 4.6: Original chart recordings of (A) Collision induced dissociation chromatogram of celecoxib and (B) the corresponding mass spectrum of authentic standard of celecoxib acquired in the negative ion mode. These original traces are typical of 100 or more such different experiments.

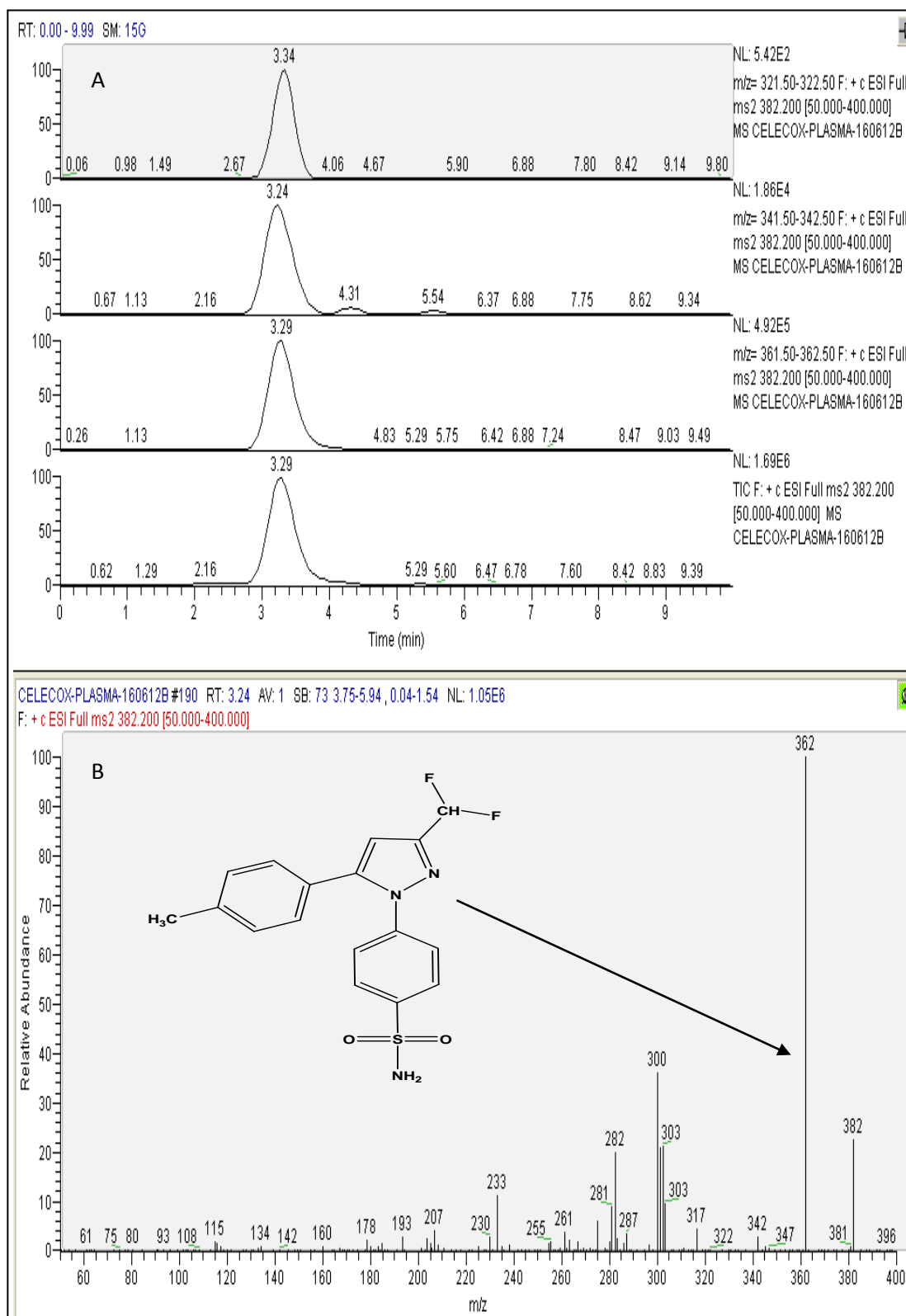


Figure 4.7: Original chart recordings of (A) collision-induced dissociation chromatograms of celecoxib and (B) the corresponding mass spectrum of authentic standards of celecoxib acquired in the positive ion mode. These chromatograms are typical of 100 or more such experiments.

fragments of m/z 366, 302, 282, 262 and 242. A loss of 44 (CO_2) from the parent drug forms 366, while a loss of 64 (SO_2) from 366 forms 302. Similarly, a further sequential loss of 20 (HF) forms 282, 262 and 242. These results have clearly confirmed that the M2 metabolite is COOH -celecoxib and is derived mainly from the basic structure of celecoxib.

Metabolite M1 – The $(\text{M}-\text{H})$ ion of M1 shown in Figure 4.9 has a mass unit of 16 greater than that of the parent compound celecoxib. This suggests that it could be a hydroxylated metabolite since it has a m/z value of 396 and therefore is hydroxymethyl celecoxib. Hydroxymethyl celecoxib could not be detected in faeces samples.

The results of this study have also shown that a neutral loss scan for m/z 176 with either urine or faeces did not give any indication for the presence of glucuronide metabolites for celecoxib in horses.

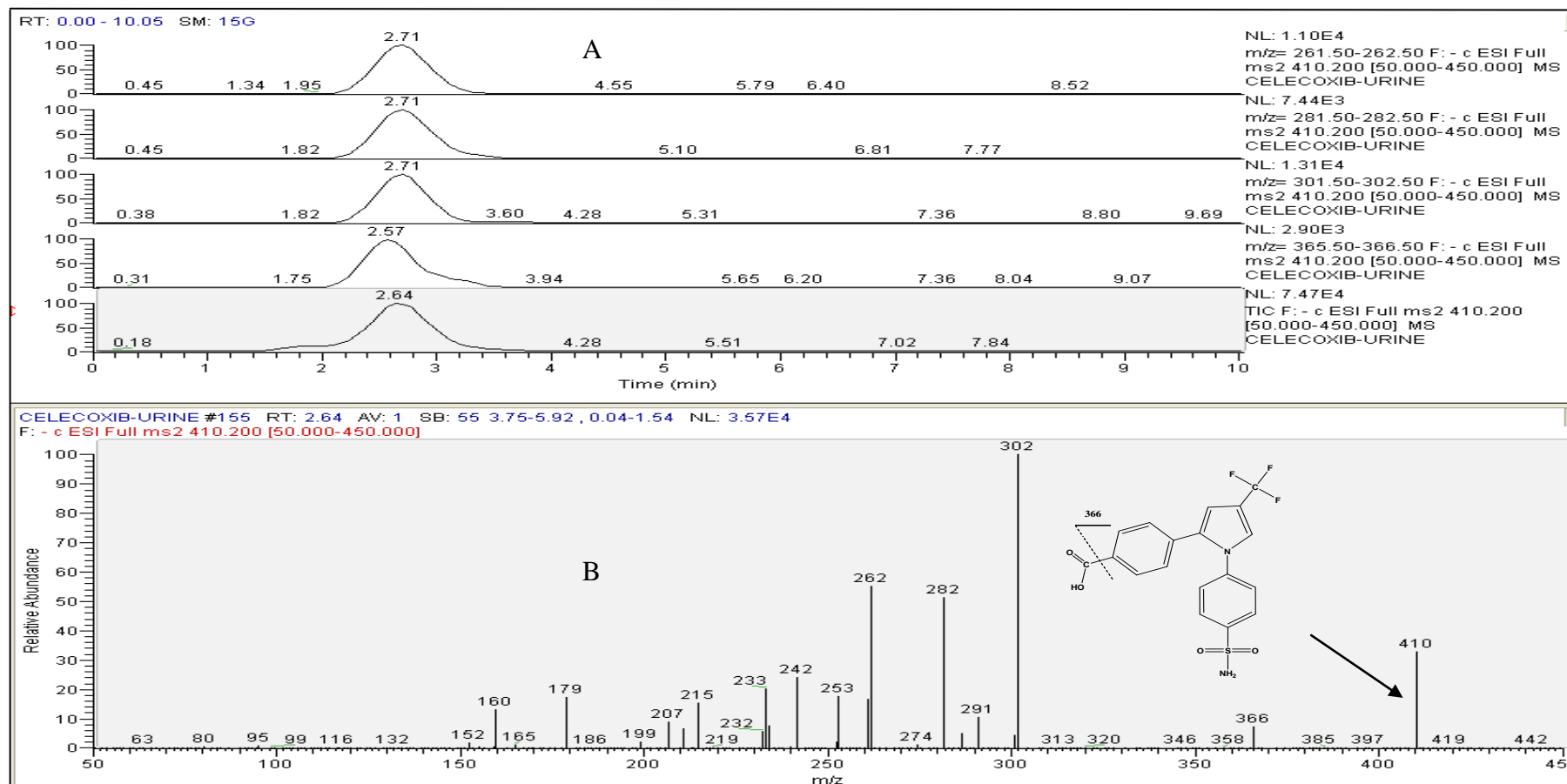


Figure 4.8: Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of carboxylic acid metabolite acquired in negative ion mode of celecoxib obtained from urine samples 24 hours post administration . These chromatograms are typical of 100 or more such experiments.

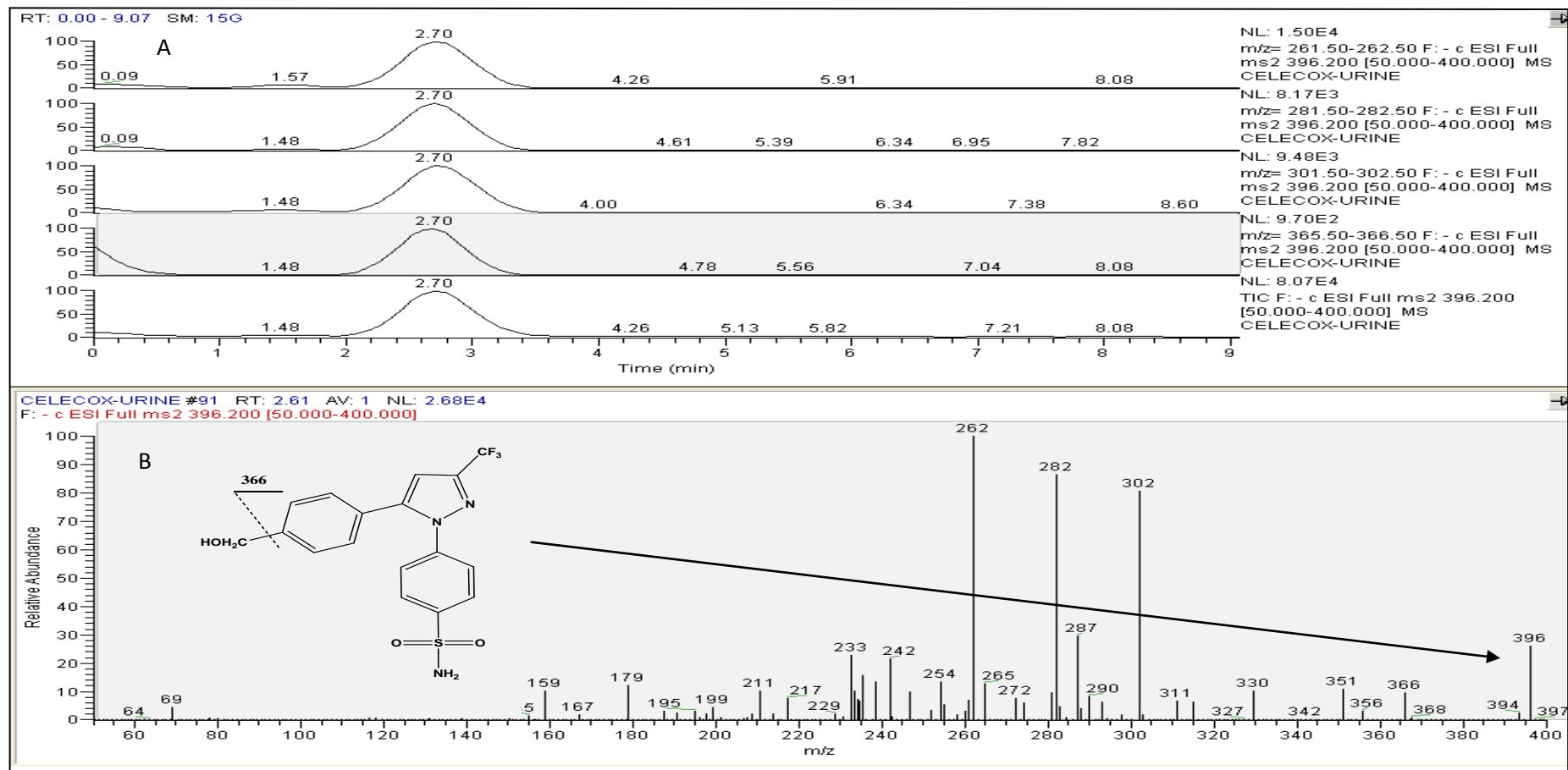


Figure 4.9: Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of hydroxymethyl metabolite acquired in negative ion mode of celecoxib obtained from urine samples after 24 hours post administration. Data are typical in excess of 6 or more such experiments.

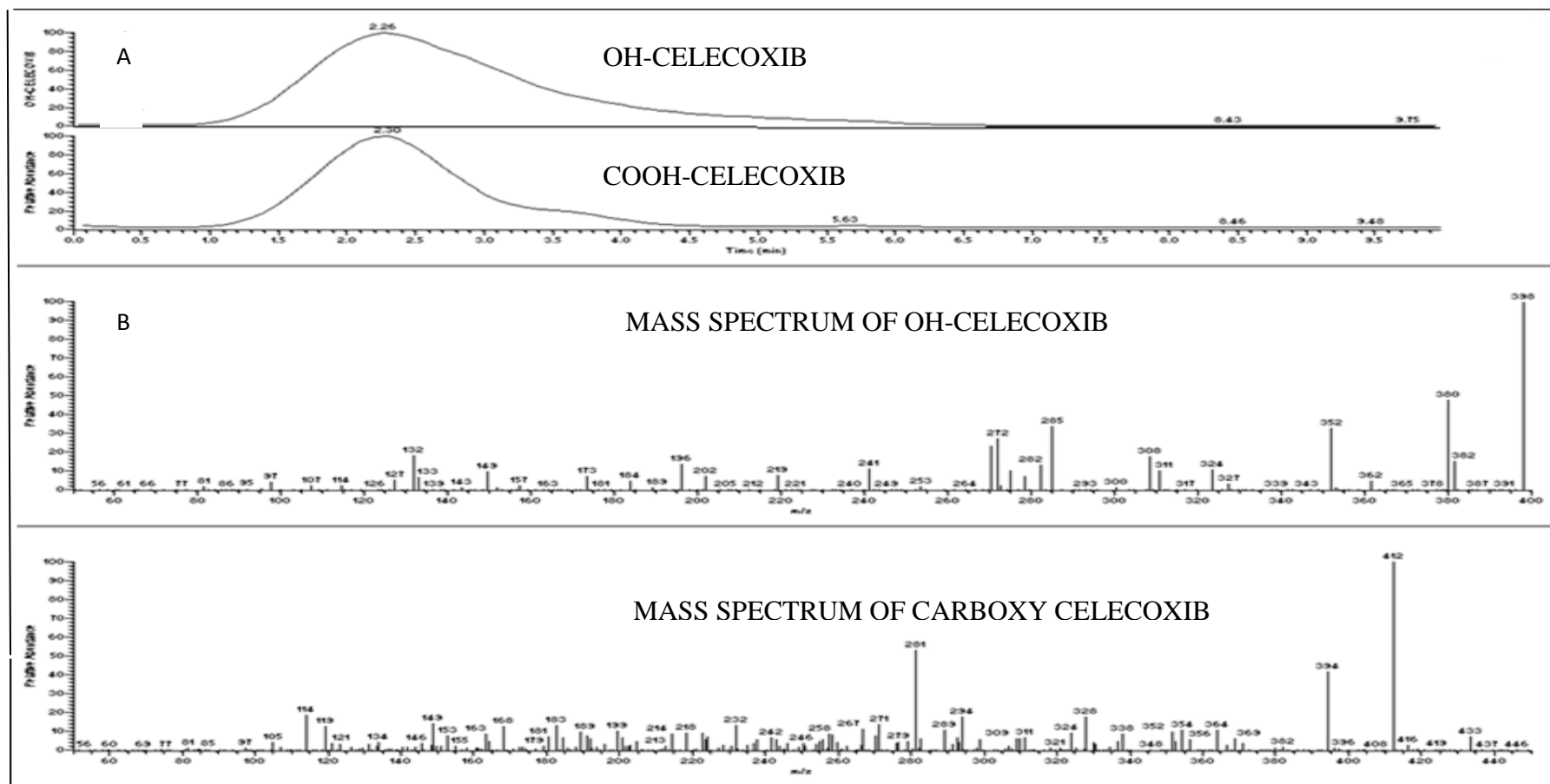


Figure 4.10: Original chart recordings showing (A) chromatogram and (B) mass spectrum of hydroxyl celecoxib and carboxyl celecoxib metabolites of celecoxib acquired in positive ion mode from in urine samples after 24 hours administration.. Data are typical of 6 or more such different experiments.

4.7. Etoricoxib analytical method validation

Selectivity and Specificity

The extraction and analytical method was validated to determine the matrix effect, sensitivity and stability by extracting samples spiked with zaleplon as internal marker and without the drug in plasma obtained from the horses prior to the administration of etoricoxib. Figure 4.11 shows original chromatograms of plasma samples with (A) zaleplon as internal marker and no signal at the retention time of etoricoxib and (B) blank plasma sample spiked with internal standard, zaleplon and etoricoxib spiked at 10 ng/ml. The data have demonstrated that no interference from endogenous peaks was observed at the retention time of either etoricoxib or the internal marker zaleplon. The data also show that extraction of plasma using a C₈-HGX, a mixed mode cartridge does not result in a signal at the retention time of the drug studied.

Precision and Accuracy

The intra-day and inter-day precision and accuracy are summarized in Table 4.3 showing a percentage of CV, ranging from 2.40 to 6.59 and 1.03 to 6.52, respectively. The LOD and LOQ based on a good signal to noise ratio of 3:1 was 1 ng/ml and the limit of quantification was 5 ng/ml.

Table 4.3: Within and inter-day variability of the LC/MS assay for determination of etoricoxib in three different concentrations in plasma (n=5). Data are mean \pm SD.

C (ng/ml)	Intra-day variability		Inter-day variability	
	Mean \pm SD	CV%	Mean \pm SD	CV%
10	9.75 \pm 0.64	6.59	10.03 \pm 0.65	6.52
100	102.04 \pm 4.67	4.67	100.97 \pm 3.93	3.90
200	201.32 \pm 4.82	2.40	199.63 \pm 2.06	1.03

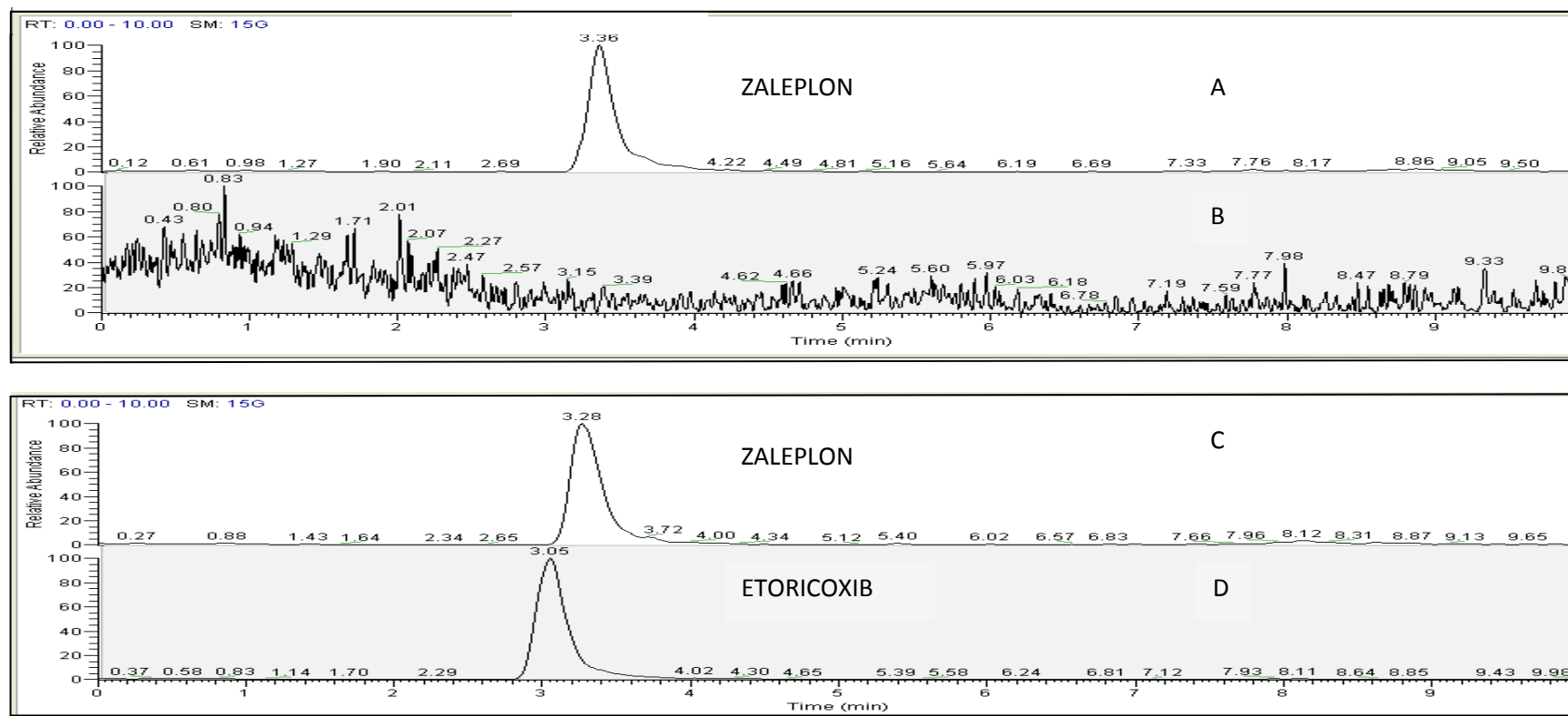


Figure 4.11: Original chart recordings of chromatograms showing (A) the retention time of zaleplon (IM), (B) the absence of any signal at the RT of etoricoxib, (C) the peak for zaleplon (IS) and (D) the peak for etoricoxib spiked in blank plasma. Chromatograms are typical of 6 such different experiments employing 6 different horses.

4.8. Pharmacokinetics of etoricoxib

The results of the pharmacokinetic analysis following oral administration of etoricoxib at either 0.5 mg/ kg, 1 mg/kg or 2 mg/kg are summarized in table 4.4. The pharmacokinetic parameters were estimated using non compartmental method employing in-house developed programme to estimate the pharmacokinetic parameters of etoricoxib

Prior to oral administration of etoricoxib, all the animals were healthy and they show no sign of distress as described in chapter 3 of this study. The results show in table 4.4 that after oral administration of etoricoxib at a concentration of either 0.5, 1 or 2 mg/kg b wt, an average maximum concentration (C_{max}) was found to be 383.72 ± 72.93 ng/ml, 512.46 ± 67.80 ng/ml and 975.84 ± 97.98 ng/ml, respectively. The absorption of etoricoxib appears to be rapid, with the time to reach peak plasma concentration (T_{max}) was just 1.0 ± 0.51 hr, 0.82 ± 0.11 hr and 0.79 ± 0.1 hr for the three different doses, respectively. The extent of drug absorption which was characterized by the area under the plasma concentration-time from zero to infinity ($AUC_{0-\infty}$) was shown to be $1,289.87 \pm 666.8$ ng/ml/hour, $4,184 \pm 1,275.32$ ng/ml/hour and $5,697.14 \pm 4,566.78$ ng/ml/hour for the 3 doses, respectively. The mean (\pm SEM) plasma concentration-time profile after oral administration of etoricoxib at a dose level of either 0.5 mg/kg, 1 mg/kg or 2 mg/kg to the six healthy retired race horses is also given in Figure. 4.11 for comparison. The results show a rapid increase in etoricoxib for each dose, reaching a maximum within 1 hour of oral administration and then decline slowly to basal levels within 5 – 40 hours depending on the concentration. Chart showing actual values and calculations performed for the three doses (0.5, 1 and 2 mg/kg bwt of etoricoxib is shown in Appendix (table 4, 5 and 6).

Table 4.4: Calculated pharmacokinetic parameters of etoricoxib following oral administration of either 0.5 mg/kg, 1 mg/kg or 2 mg/kg bwt. Data mean \pm SD, n=6

Parameters	(Mean \pm SD)		
	0.5 mg/kg	1 mg/kg	2 mg/kg
C _{max} (ng/ml)	383.72 \pm 72.93	512.46 \pm 67.81	975.84 \pm 97.98
K	0.07 \pm 0.03	0.09 \pm 0.01	0.06 \pm 0.01
t _{max} (hours)	1 \pm 0.51	0.82 \pm 0.11	0.79 \pm 0.1
t _{1/2} hours	10.06 \pm 3.73	8.30 \pm 1.44	11.51 \pm 1.56
AUC _{total} (ng/ml/hr)	1,289.87 \pm 666.8	4,184 \pm 1275.32	5,697.14 \pm 4566.78
C ₍₀₎	197.79 \pm 82.95	291.60 \pm 94.85	628.42 \pm 50.85

4.9. Metabolism and excretion of etoricoxib in plasma, urine and faeces

Six horses were administered etoricoxib and the concentrations of etoricoxib and its metabolites were determined in plasma, urine and faeces samples over time. Etoricoxib was administered at a concentration of either 0.5, 1 or 2 mg/kg b wt and the plasma excretion profile was studied at all the concentrations. A positive ion mode LC/MSMS was employed for the detection of etoricoxib and its metabolites in preference to less sensitive negative ion mode analysis.

Plasma: The mean concentration-time profiles of etoricoxib in plasma following administration of either 0.5, 1 or 2 mg/kg b wt are shown in Figure.4.12. The results show that etoricoxib levels increase rapidly reaching 383.72 ± 72.93 ng/ml, 512.46 ± 67.81 ng/ml and 975.84 ± 97.98 ng/ml in less than or approximately an hour for the three different doses. Additionally, 6'-hydroxymethyl etoricoxib with m/z 375 (M+H) and with m/z of 16 greater than the administered parent etoricoxib was detected in plasma. These results are shown in Figure.4.13 and they accounted for about 80% of the concentration of etoricoxib. Similarly, mass spectral identification made it possible to quantify the concentration of the metabolite formed from etoricoxib. Pure metabolite standards are not commercially available, hence, as shown in Figure.4.13, and based on the mass spectral identity, quantification of 6'-hydroxymethyl etoricoxib showed a concentration of 364.31 ± 36.48 ng/ml (mean \pm SEM) after an oral administration of 1 mg/kg b wt of etoricoxib to the 6 horses. The concentration of 6'-hydroxymethyl etoricoxib peaked at around 3 hours after oral administration. In agreement with data presented in Figure 4.13, etoricoxib was the major component from the first time point through 24 hours post administration sample of etoricoxib. Plasma detection of etoricoxib was possible for up 72 hours, while 6'-hydroxymethyl etoricoxib was

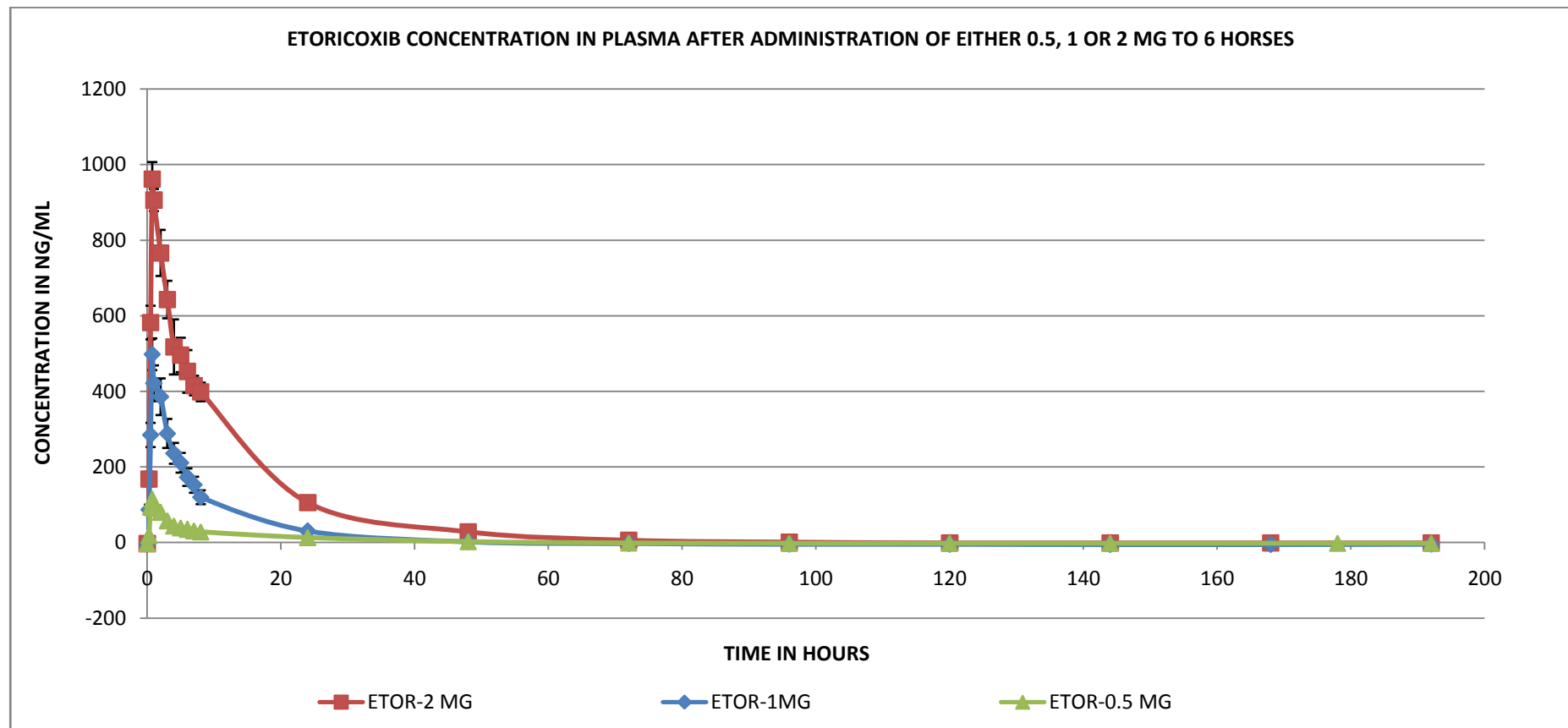


Figure 4.12: Time course plasma excretion curves of etoricoxib given as an oral administration at a dose of either 0.5, 1 or 2 mg/kg b wt. Data are mean \pm SEM, n=6. Note that etoricoxib is absorbed rapidly after administration and decreases slowly to reach a non-detectable level at 50-60 hours.

detected for a longer period, estimated to be about 96 hours. It can be concluded from these results that plasma may be a better matrix for the screening and the detection of etoricoxib or its metabolites in the horse racing industry in order to determine any form of illegal use of the drug prior to the race.

Urine: Similar to celecoxib, solid phase extraction of unhydrolysed urine followed by mass spectrometry revealed etoricoxib to be present in low concentration and thus indicating extensive metabolism. Investigating the metabolism of etoricoxib using product ion scan in a triple stage quadrupole instrument showed peaks for possible hydroxylation, carboxylation and oxidation (M3) of the parent etoricoxib. Etoricoxib presented characteristic fragmentation at two sites of the molecule. These two losses were found to be a fragmentation signature of etoricoxib. In unhydrolysed urine, an entity corresponding to the oxidation of etoricoxib was observed to have a m/z of 375 (M+H) which co-eluted with another huge peak as shown in Figure 4.14. This peak was a sixteen mass unit greater than etoricoxib and it is similar to the metabolite identified in plasma indicating the formation of 6'-hydroxymethyl etoricoxib. Furthermore, a peak of m/z 389 indicated the formation of a 6'-carboxy etoricoxib and another peak with a m/z charge of 391 indicating an oxidation and subsequent formation of 6'-hydroxymethyl-etoricoxib-1'-N-oxide.

Since it was not clear about the huge peak co-eluting with 6'-hydroxymethyl etoricoxib, hydrolysis of urine with β - glucuronidase at 37° C overnight, showed a m/z 551 in mass spectrometry. This peak has m/z 176 greater than that of the hydroxymethyl etoricoxib metabolite and thus, could be a hydroxymethyl glucuronic acid metabolite of etoricoxib. Neutral loss scan for a loss of m/z 176 yielded m/z 375 which further confirmed the formation of a hydroxymethyl glucuronic acid metabolite of etoricoxib.

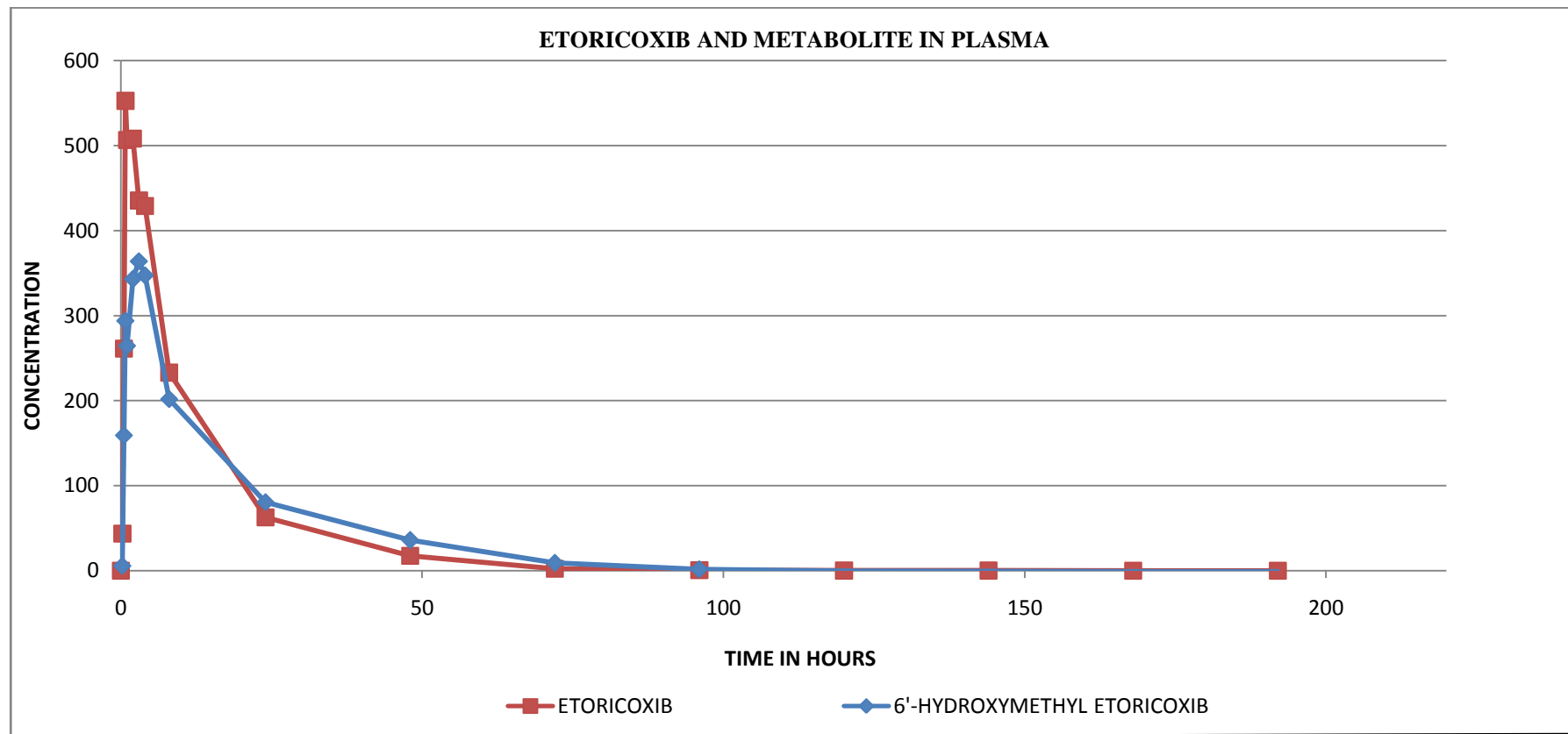


Figure 4.13: Time course changes in the levels of etoricoxib (solid triangles) and its metabolite, OH-methyl etoricoxib (solid squares) in plasma for horses. Data are mean \pm SEM, n=6. Note the rapid increases of both substances within the first few hours after oral administration and also their rapid decline within a day of administration. The values decreased to almost zero level after 80 - 90 hours following oral administration.

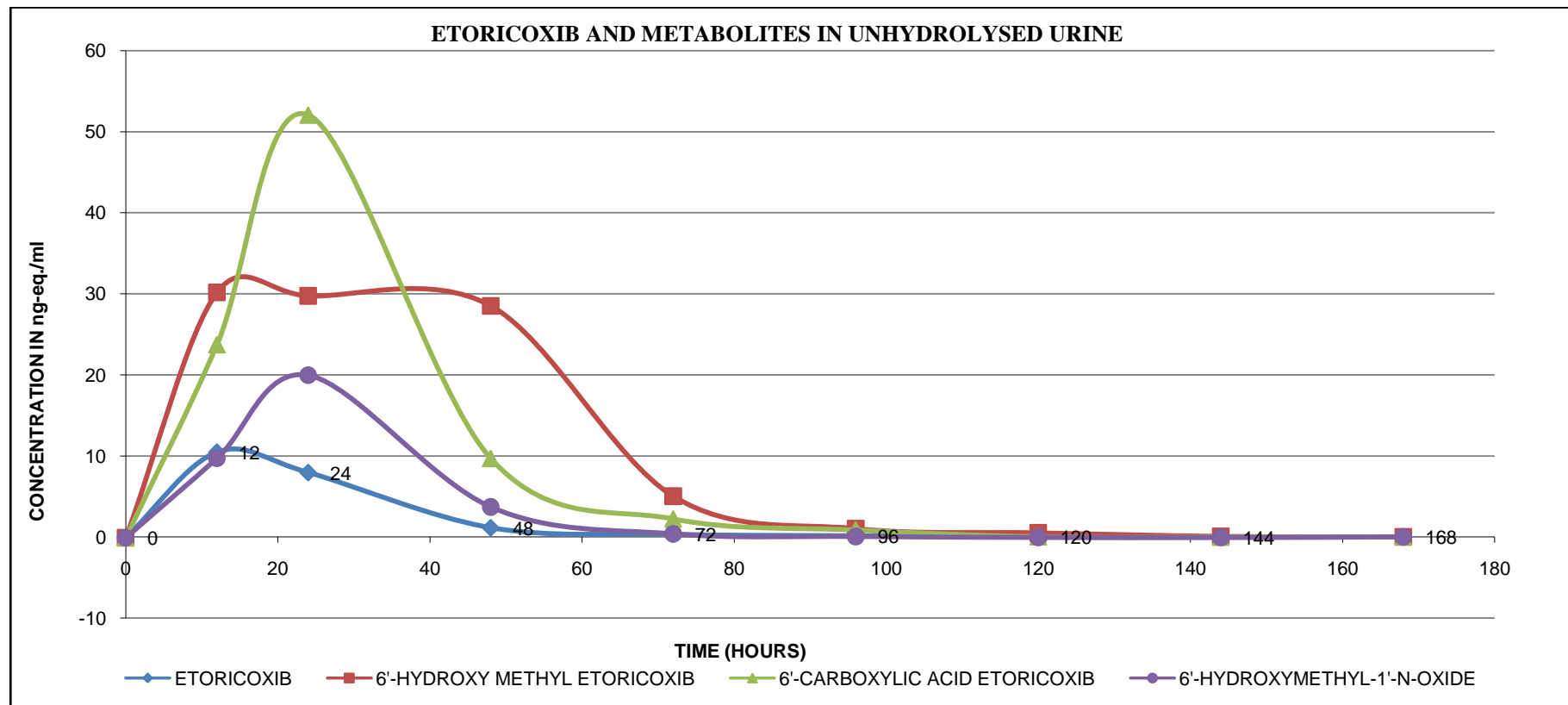


Figure 4.14: Time course changes in levels of etoricoxib (solid diamond), OH-methyl etoricoxib (solid squares) and COOH-etoricoxib (solid triangles) and etoricoxib – 1-N-oxide (solid circles) in urine samples. Note the large excretion of COOH-etoricoxib in urine compared to OH-methyletoricoxib or etoricoxib.

The data presented in Figure 4.14 reveal that etoricoxib is apparently undergoing extensive metabolism in urine giving a value of about 10 ng/ml in approximately 12 hours and subsequently, reaching to non detectable levels within 60 hours after administration of 1 mg/kg b wt of etoricoxib. The metabolites were quantified and based on the masses and the results suggest that 6'-hydroxymethyl etoricoxib was the major metabolite in urine showing a concentration of 310.13 ± 48.11 ng/ml (mean \pm SEM) at 4 hours post administration. This was followed by 6'-carboxylic acid etoricoxib which was detected at a concentration of 149.52 ± 24.05 ng/ml (mean \pm SEM) after 8 hours post administration. The data in Figure 4.15 further show that incubation of urine with β -glucuronidase can result in a decrease in the concentration of the 6'-carboxylic acid etoricoxib and a concomitant increase in the level of 6'-hydroxymethyl etoricoxib. The results also show that 6'-hydroxymethyl-etoricoxib-1'-N-oxide and the unchanged etoricoxib were found to be less than 10%.

Faeces: Figure 4.16 shows the time course excretion profile of etoricoxib and its metabolites in faeces. The results show that 6'-hydroxymethyl etoricoxib is seen in large amounts and similar to urine, is the major metabolite seen in faecal samples of horses. The 6'-hydroxymethyl etoricoxib metabolite concentration in faeces peaks at about 2 days to give a value of 78.20 ± 6.56 ng/ml (mean \pm SEM) and this is detected for up to 5 days after oral administration of etoricoxib to the 6 horses. This is followed by 6'-carboxylic acid etoricoxib seen in a concentration of approximately 32.69 ± 6.53 ng/ml (mean \pm SEM) and it is detected for about 4 days. In contrast, etoricoxib and 6'-hydroxymethyl-etoricoxib-1'-N-oxide were detected in very low levels for up to 3 days. There was no evidence for the presence of any glucuronic acid conjugate of 6'-hydroxymethyl etoricoxib.

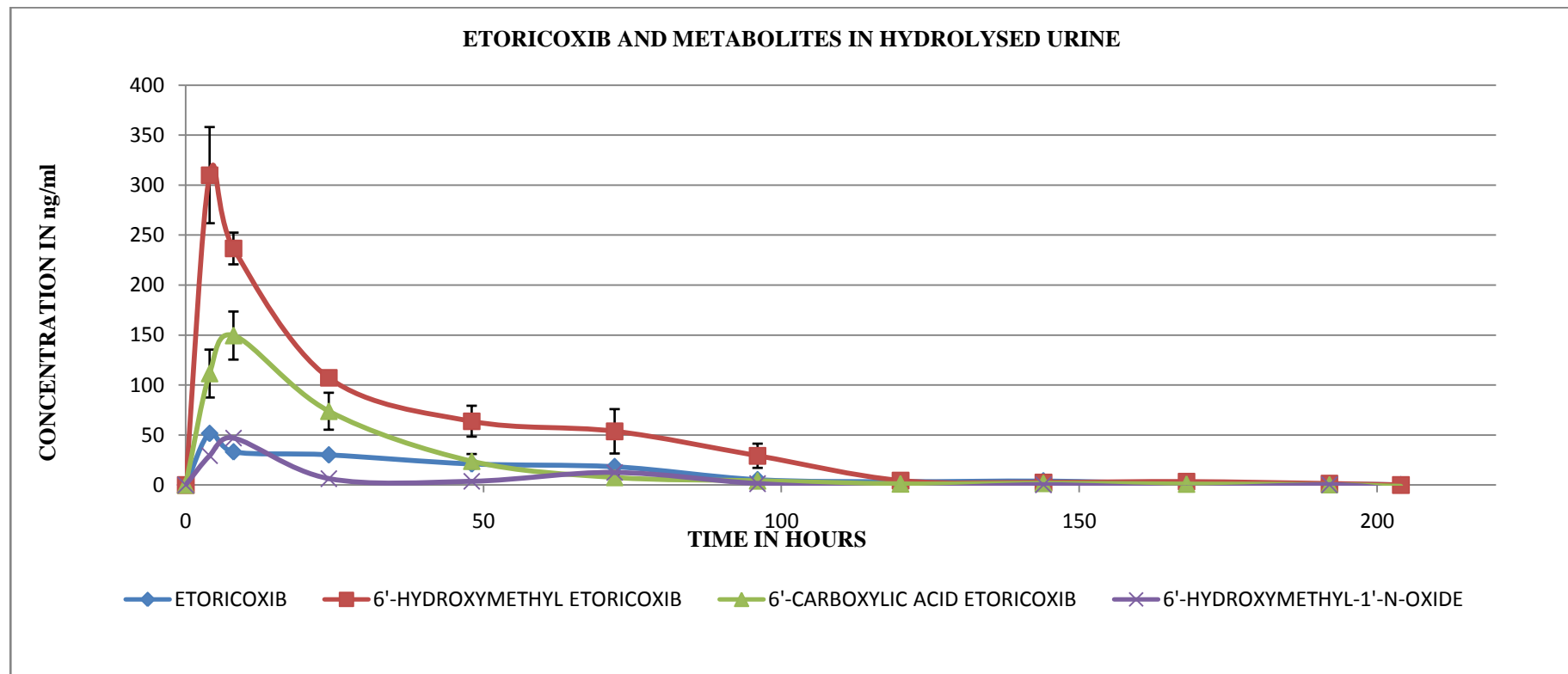


Figure 4.15: Time course of hydrolyzed urine excretion of etoricoxib and its metabolites. Data are mean \pm SEM, n=6. Note that 6'-hydroxymethyl etoricoxib peaks at around 4 hours and is detected up to 120 hours after oral administration of etoricoxib to the six horses.

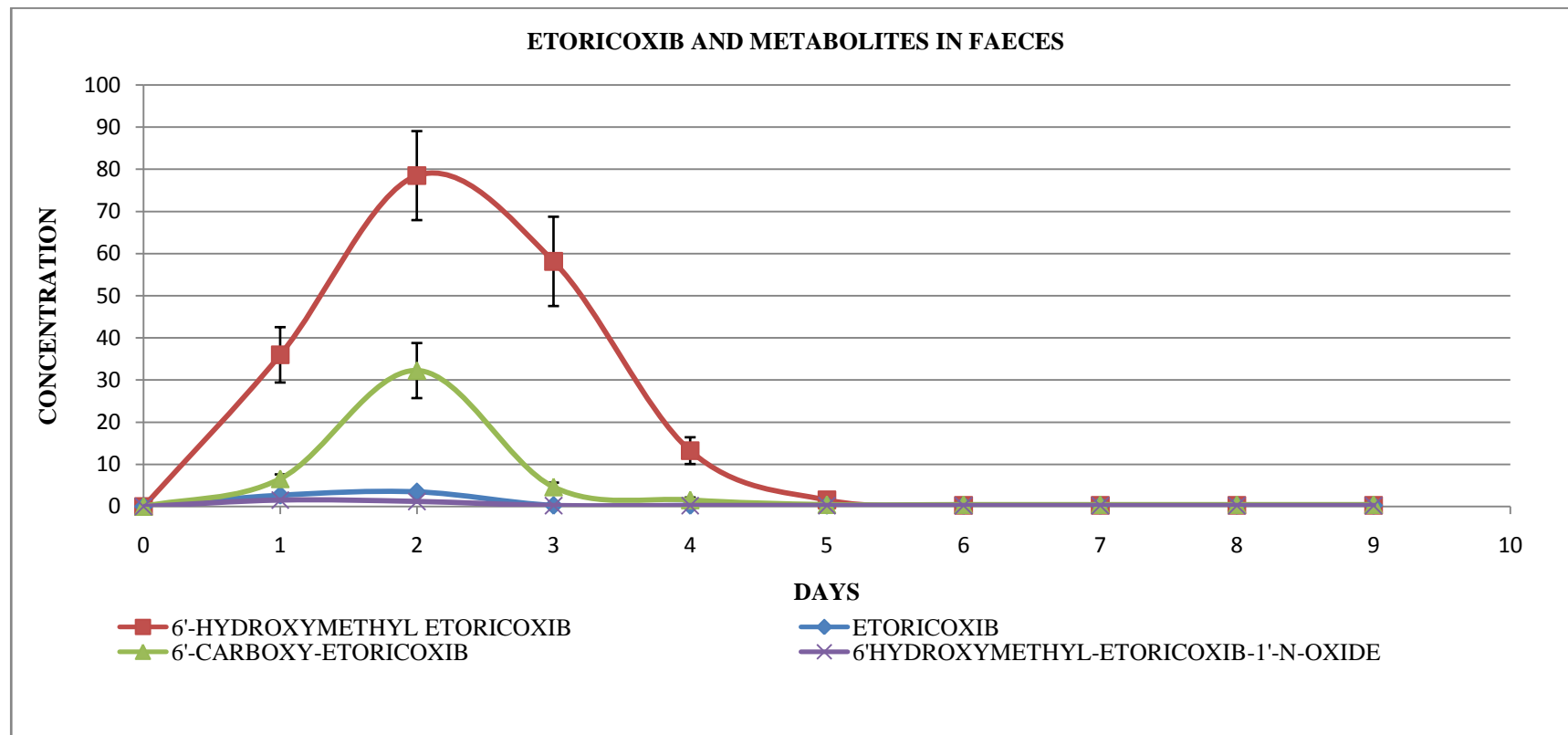


Figure 4.16: Time course of hydrolyzed faecal excretion of etoricoxib and its metabolites. Data are mean \pm SEM, n=6. Note that 6'-hydroxymethyl etoricoxib and its 6-hydroxymethyl-etoricoxib metabolite were almost un-dectable in the faecal samples. In contrast, 6-hydromethyl-etoricoxib and 6-carboxy-etoricoxib peak at 2 hours and they were detected for around 4 and 5 hours, respectively in the samples of the 6 horses.

4.10. Identification of hydroxymethyl, carboxylic acid and hydroxymethyl-eticorcoxib-1'-N-oxide metabolite of etoricoxib

The metabolism of etoricoxib after an oral administration of 1 mg/ kg b wt to 6 horses shows that etoricoxib is extensively metabolized with less than 10% is excreted unchanged and is broken down into 6'-hydroxymethyl etoricoxib, 6'-carboxylic acid etoricoxib, 6'-hydroxymethyl-eticorcoxib-1'-N-oxide and glucuronide of 6'-hydroxymethyl etoricoxib.

Figure 4.17 shows the mass chromatogram of etoricoxib and the corresponding mass spectrum acquired in the positive ion mode and having a m/z value of 359 ($M+H$) which on fragmentation loses a m/z 79 (SO_2CH_3) to give a m/z 280. A m/z of 375 for hydroxymethyl etoricoxib with its characteristic loss of 79 as a fragment of m/z 296 is shown in Figure 4.18. In addition, 6'-hydroxymethyl etoricoxib has also been identified in the plasma of the horses following oral administration of etoricoxib. Figure 4.19 shows a peak of m/z 551, typical of a mass of glucuronic acid metabolite of 6'-hydroxymethyl etoricoxib. The formation of hydroxymethyl etoricoxib from the glucuronide of hydroxymethyl etoricoxib following enzyme hydrolysis of urine samples and confirmed by mass spectrometry, loses 176 Da to form a peak of m/z 375. The results suggest that there was no evidence of the glucuronic acid metabolite of 6'-hydroxymethyl etoricoxib in faeces of the horses following oral administration of etoricoxib. This observation was substantiated by mass spectrometry measurement of either unhydrolyzed or hydrolyzed faecal samples of the etoricoxib. There is a greater chance that the glucuronic acid conjugate is hydrolyzed by the gut bacteria in the intestine of the animal. Thus, 6'-hydroxymethyl etoricoxib, both free and conjugated forms, are the major metabolite in horse urine. Figure 4.20 shows the extracted ion

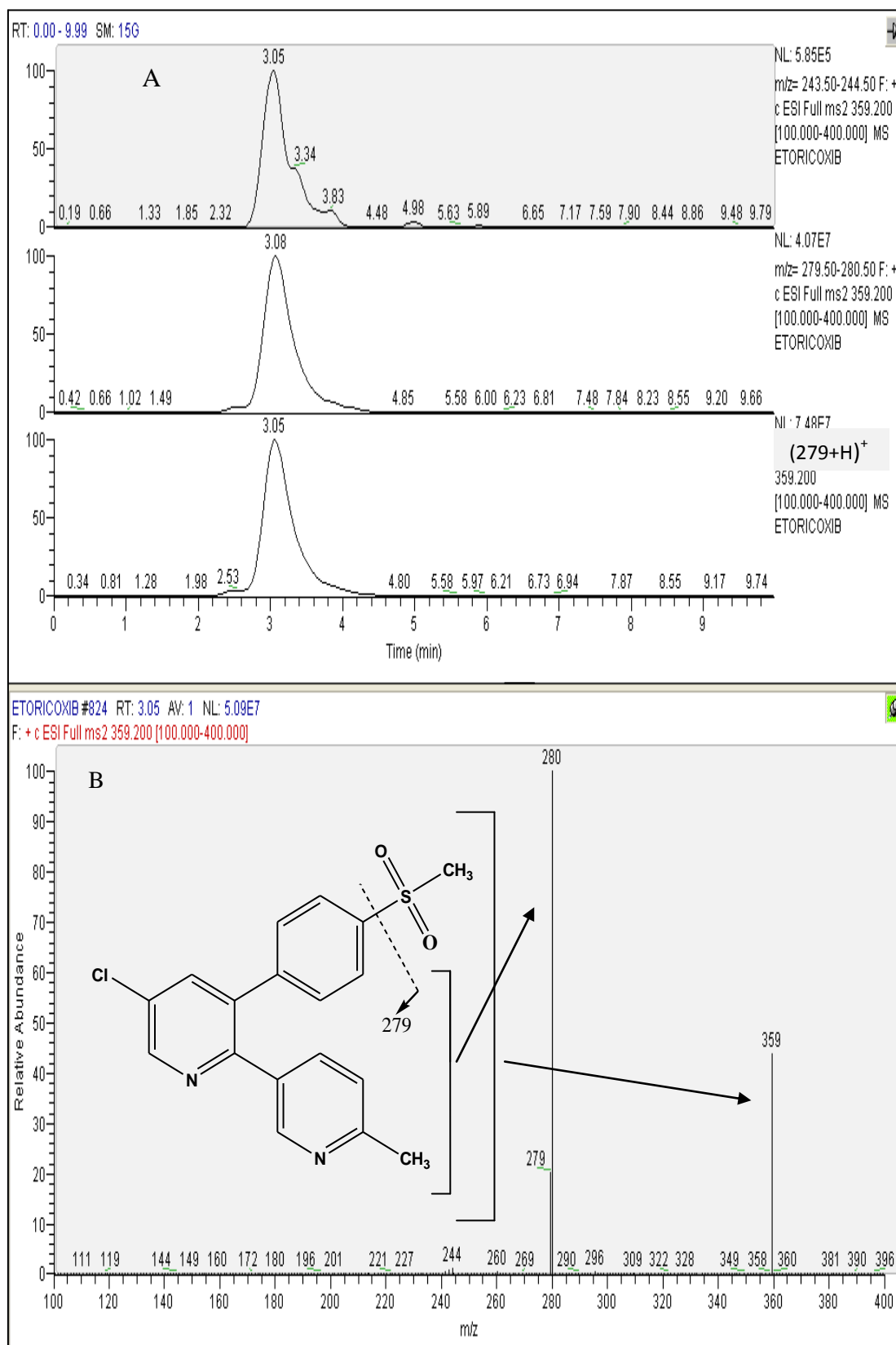


Figure 4.17: Original chart recordings showing (A) collision- induced dissociation chromatograms of etoricoxib and (B) the corresponding mass spectrum of authentic standard of etoricoxib acquired in the positive ion mode. These chromatograms are typical of 100 or more such experiments.

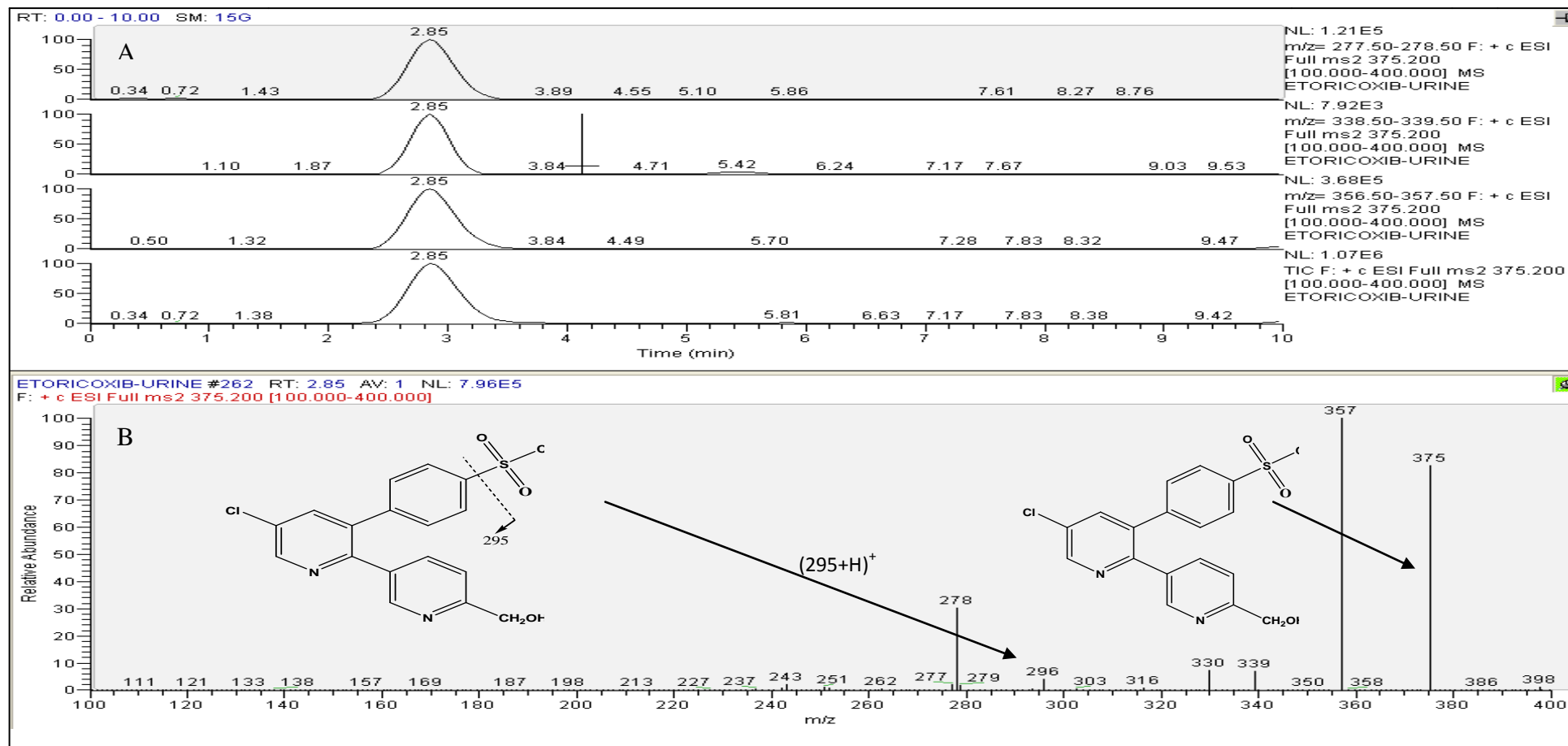


Figure 4.18: Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of 6'-hydroxymethyl metabolite acquired in positive ion mode of etoricoxib obtained from hydrolyzed urine samples 24 hours post administration. Data are typical in excess of 50 or more such experiments.

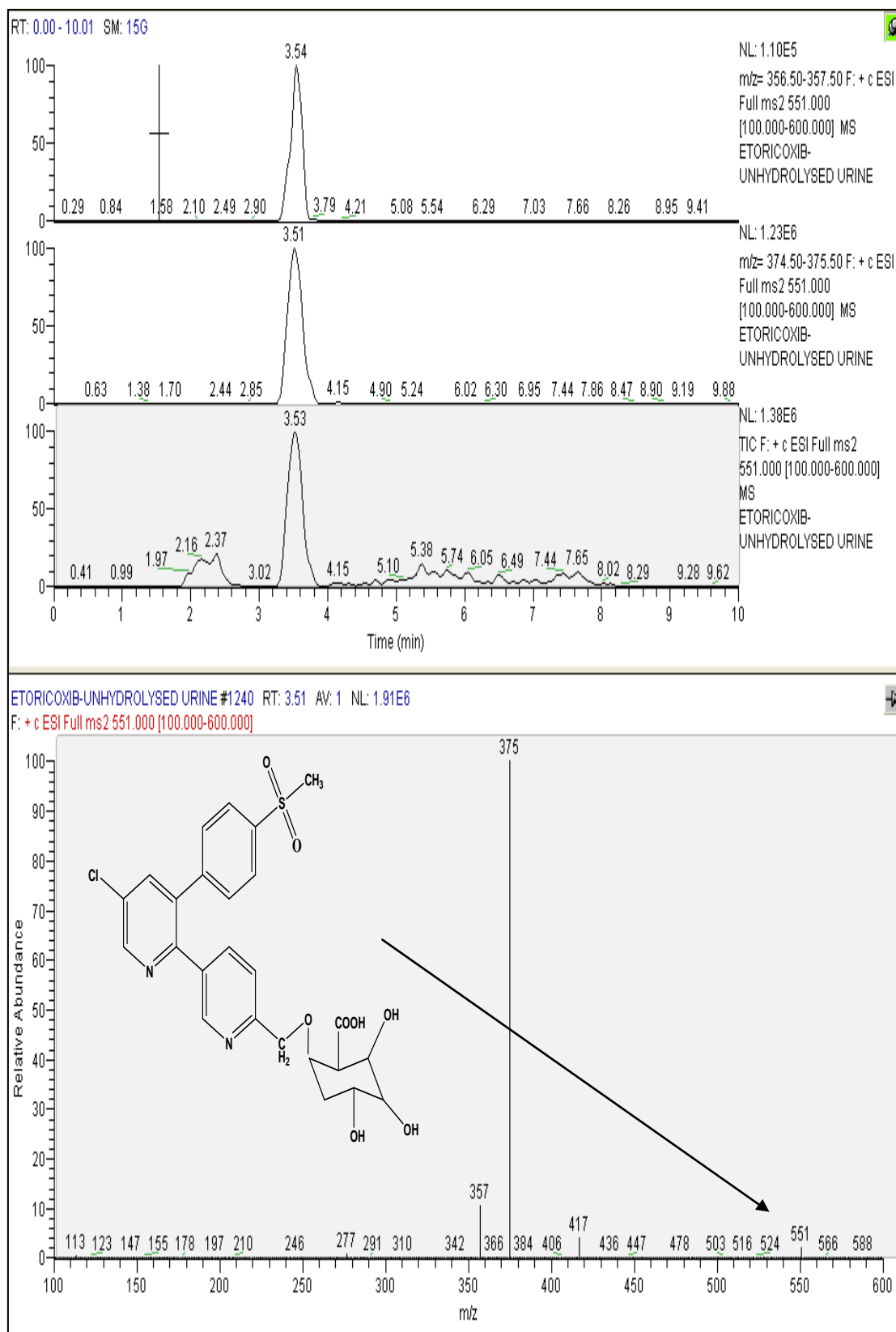


Figure 4.19: Original chart recording showing (A) extracted ion chromatograms and (B) mass spectrum of glucuronic acid metabolite of 6'-hydroxymethyl etoricoxib acquired in positive ion mode and obtained from urine samples 24 hours post administration. These chromatograms are typical of 50 or more such experiments.

chromatogram and the corresponding mass spectrum of m/z 389, which is most likely to be a carboxylic acid metabolite of etoricoxib.

The 6'-hydroxymethyl etoricoxib further undergoes oxidation to form the 6'-carboxylic acid metabolite of etoricoxib. The 6'-carboxylic acid etoricoxib is not present in horse plasma. Figure 4.21 shows the extracted ion chromatogram and the corresponding mass spectrum of m/z 391 typical of N-oxidation of 6'-hydroxymethyl etoricoxib to form 6'-hydroxymethyl etoricoxib-1'-N-oxide. This metabolite was found in very low levels both in urine and faeces. Although authentic pure standards for 6'-hydroxymethyl etoricoxib, 6'-carboxylic acid etoricoxib, 6'-hydroxymethyl etoricoxib-1'-N-oxide and glucuronic acid metabolite of 6'-hydroxymethyl etoricoxib were not available in this study, it was nevertheless still possible to identify their presence either in urine or faeces. This was done with some certainty based on their mass to charges and the corresponding mass spectrum.

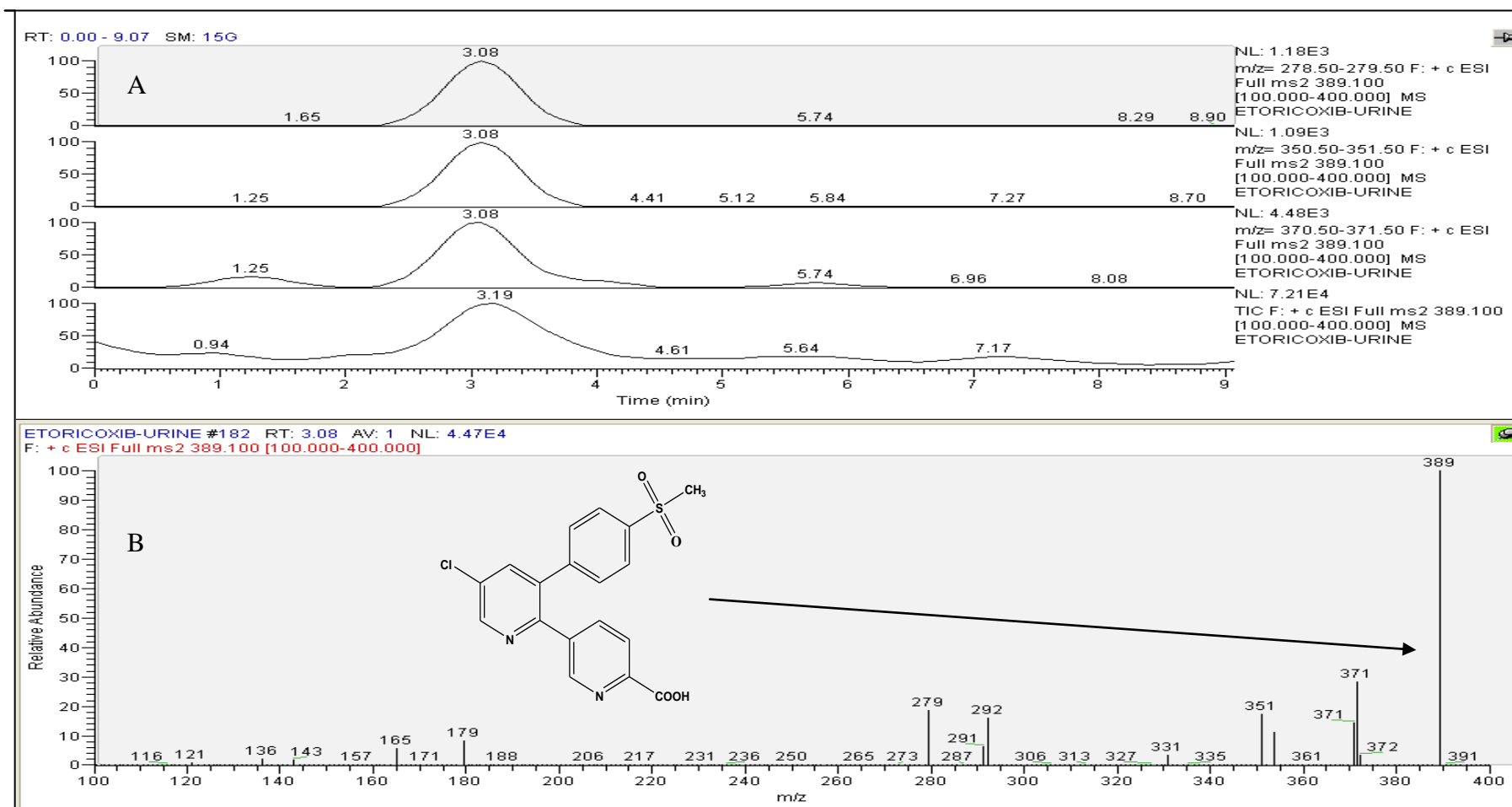


Figure 4.20: Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of 6'-carboxylic acid metabolite of etoricoxib obtained from urine samples from horses. Traces are typical of over 50 such different experiments.

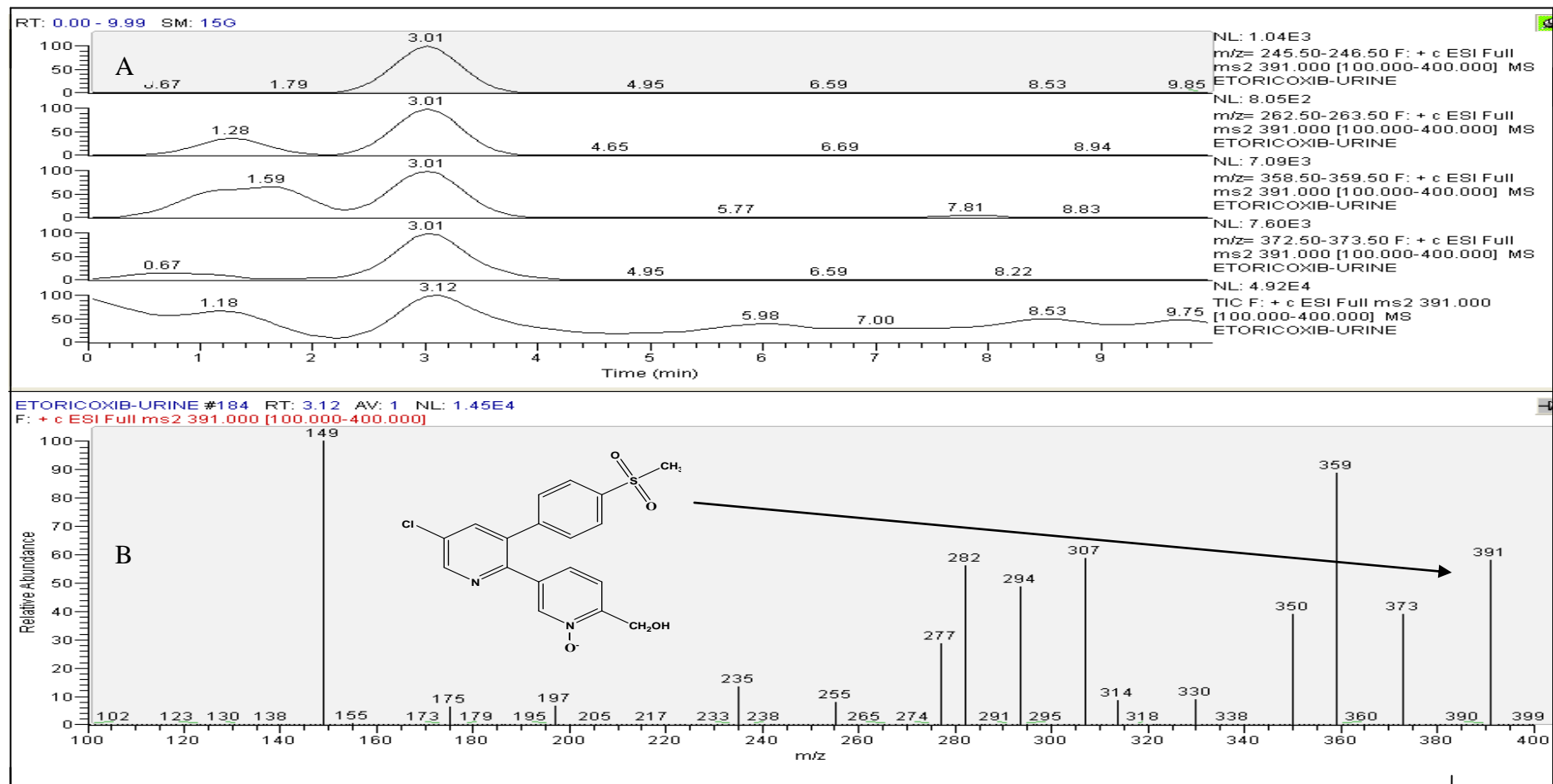


Figure 4.21: Original chart recordings showing (A) extracted ion chromatograms and (B) mass spectrum of hydroxymethyl-1'-N-oxide metabolite of etoricoxib in urine samples from horses. These traces are typical of more than 50 such different experiments.

4.11. Discussion

The development of pain, especially in the horse racing industry, is a major problem for race horses and their owners. The normal or traditional way in treating the pain is to give the horses oral doses of non-steroidal anti-inflammatory drugs (NSAIDs). There are different types of NSAIDs on the market and many of them have adverse side effects especially gastric ulcers (Ferreira *et al.*, 1973; Vane, 1994).

Recently, safer NSAIDs called coxibs have been developed to treat pain in human as well as other animals. To date, not much study has been done on the metabolism of the coxibs in large animals such as camels and horses which are normally used in the racing industry. Therefore, the main purpose of this study was to investigate the metabolism of two commercially available coxibs namely, celecoxib and etoricoxib, measuring their absorption and disposition in six retired race horses following oral administration of either 2 mg/kg b wt of celecoxib or either 0.5, 1 or 2 mg/kg b wt of etoricoxib.

The rationale for this study is to help, not only the Veterinarians who look after the health of the horses, but also the racing industry fraternity, including the owners of the race horses about the possible withdrawal times when using these two COX-2 inhibitors in horses for the treatment of osteoarthritis or pain. This study also attempts to enhance knowledge and understanding of the possible metabolites derived from celecoxib and etoricoxib following their metabolism and moreover, their detection times in biological samples. In turn, the results may provide vital information to the racing forensic laboratory in both detecting and preventing the misuse of performance enhancing substances like celecoxib and etoricoxib on a race day. In this study, the six retired race horses were given orally either celecoxib or etoricoxib at physiological doses and the fate (metabolism and by products or metabolites) of each drug was analyzed in plasma,

urine and faecal samples over duration of 5 days employing established LS/MS techniques.

The results of this study show that all six animals were able to tolerate the two NSAIDs favourably without any side effects (see chapter 3) and it was not difficult to obtain the urine, blood and faecal samples for measurements. For analysis, the ion efficiency of celecoxib in the positive ion and negative ion mode was tested by infusing 10 ng/ml at a flow rate of 5 μ l/min. The positive ion mode fragmentation of celecoxib yielded only one fragment of m/z 362, which is a loss of m/z 20. No other ions fragments were formed for celecoxib and hence, positive ion mode fragmentation was found not characteristic for detection of celecoxib. The present results also show that the negative ion mass spectrum of celecoxib and its metabolites produced three fragments with loss of m/z 20 corresponding to hydrogen fluoride (HF), each of which was found to be very characteristic of celecoxib and its metabolites in identification and interpretation of the mass spectrum data.

The pharmacokinetics of celecoxib after an oral administration of 2 mg/ kg b wt shows that C_{\max} was $1,157.87 \pm 323.58$ ng/ml, t_{\max} was 4.09 ± 1.60 hours and $t_{1/2}$ was 25.52 ± 8.40 hours. These pharmacokinetic parameters obtain in this study are in full agreement and in accordance with the parameters reported in other animal species, including dogs (Paulson *et al.*, 2001). In their study on celecoxib metabolism in dogs following oral administration, Paulson et al (2001) found the t_{\max} to be 3.3 ± 1.7 hours and $t_{1/2}$ to be around 5.1 ± 0.5 hours. In another study, Itthipanichpong *et al.*, (2005) administered celecoxib orally to Thai human volunteers and they showed that the peak plasma concentration of the coxib reached within 2.55 ± 1.22 hours and the elimination half life was 8.79 ± 5.49 hours. Similarly, Emami *et al.*, (2008) have reported a t_{\max} of $1.87 \pm$

0.18 and a $t_{1/2}$ of 12.76 ± 1.44 hours in their study when celecoxib was administered orally to 12 healthy male volunteers. Together, the present pharmacokinetic data obtained from horses in this study and those previously reported in the literature employing either dogs (Paulson et al, 2001) or human ((Itthipanichpong *et al.*, 2005) are more or less in complete agreement for celecoxib irrespective of species differences.

The results presented in this study have demonstrated that after an oral administration of celecoxib to 6 healthy horses, the peak plasma concentration of the drug reached about 1 $\mu\text{g/ml}$ in four hours. This was followed by a rapid decline within the first 6-8 hours and the level decreased gradually to reach almost zero level after 75-80 hours following administration. Similarly, celecoxib at a peak concentration of 287.77 ± 53.84 ng/ml was excreted in faeces after 24 hours of oral administration, followed by a rapid decline to almost zero level after 80 hours following administration. In contrast, celecoxib was found in small amount in urine, accounting for 4.8 ± 1.44 ng/ml after 24 hours following oral administration. This then declined to almost zero level after 60 hours of administration. This excretion profile of celecoxib as an unabsorbed drug in faeces suggests that the major route of excretion is probably biliary. The clearance of celecoxib in humans is about 27% in urine and 70% in faeces (Antonioniou *et al.*, 2007).

The data presented in this study show that the plasma metabolite profile of celecoxib in the six horses revealed the presence of two metabolic pathways for the COX inhibitor. The first includes the hydroxylation at the methyl group in the phenyl ring of celecoxib to form hydroxyl-methyl celecoxib, which then gets oxidized at the hydroxyl group to form carboxylic acid metabolite of celecoxib. The second includes/could be either oxidation of the methyl group or hydroxylation of the phenyl ring. Interestingly, the hydrophenyl metabolites derived from celecoxib are eliminated in rabbit plasma (Zhang

et al., 2000). The plasma metabolic profile for such animal species as mouse, dogs and rhesus and cynomolgus monkey show COOH celecoxib as the major metabolite compared to hydroxymethyl celecoxib. Moreover, they show no evidence of hydroxyphenyl metabolite (Paulson *et al.*, 2000b).

The results of this study show that following oral administration of celecoxib, only low concentration of the parent drug was eliminated in urine at a low concentration of around 4.8 ± 1.44 ng/ml and traces could still be detected for up to 60 hours suggesting extensive metabolism. COOH-celecoxib at a concentration of 105.53 ± 19.09 ng/ml was measured in urine samples following 24 hours of oral administration of celecoxib. This value declined rapidly to almost zero level following 96 hours of administration. Only small traces of hydroxymethyl celecoxib at a concentration similar to the parent celecoxib concentration was detected in the urine samples.

In the present study, an interesting observation was noticed on the metabolism of celecoxib in the six horses where the COX-2 inhibitor was the main component in the faeces. The parent drug was measured at a high concentration of about 287.77 ± 53.84 ng/ml following 24 hours of oral administration and this amount was detected for up to 72 hours following administration. In contrast, the metabolite, COOH-celecoxib was detected at a peak concentration of 91.12 ± 40.22 ng/ml following 24 hours of oral administration of celecoxib. Thereafter, COOH-celecoxib declined to almost undetectable levels 96 hours following administration. One possible explanation for the presence of a high amount of the parent celecoxib in the faeces is that the drug was not properly absorbed by the intestine of the horse. Another possible explanation is the COX-2 inhibitor is slowly metabolized in the GI tract. This is an interesting area worthy

of further research. In a previous study Paulson et al (2001) reported that COOH-celecoxib was the major

metabolite detected in faecal samples whereas hydroxymethyl celecoxib was the minor metabolite detected in urine and faeces of mouse, rabbit, dog and monkey. In addition, these authors reported that dog, cynomolgus and rhesus monkey excreted no unchanged drug (Paulson *et al.*, 2000b). In another study, Paulson et al (2000b) reported that conjugation of the hydroxyl and carboxylic acid metabolite of celecoxib with glucuronic acid can occur in the body following the metabolism of celecoxib. In contrast, the results of the present study employing the six race horses have shown no evidence of glucuronide conjugation during the metabolism and excretion of celecoxib.

The present results show that the LC/MS retention time of celecoxib is identical to that of authentic celecoxib standard. The m/z 380 and the collision- induced dissociation fragmentation pattern in the negative ion mode were consistent to the deprotonated ion of celecoxib standard. Authentic pure standards of the metabolites of celecoxib were not available commercially and syntheses of these metabolites are outside the remit of this study. However, the mass spectrum and the CID fragmentation pattern of the COOH-celecoxib, hydroxymethyl celecoxib were similar to previously published reports (Zhang *et al.*, 2000).

In addition to celecoxib, the present study investigated the pharmacokinetics of etoricoxib after an oral administration of 0.5, 1 or 2 mg/kg b wt. The results shows that C_{\max} was either 383.72 ± 72.93 ng/ml, 512.46 ± 67.81 ng/ml or 975.84 ± 97.98 ng/ml and AUC_{total} was either $1,289.87 \pm 666.8$ ng/ml/hr, 4184 ± 1275.32 ng/ml/hr or $5,697.14 \pm 4,566.78$ ng/ml/hr for the three different doses of 0.5, 1 or 2 mg/kg b wt, respectively. The results reveal that AUC_{total} and C_{\max} values increased approximately

proportionately with the increased doses. Absorption of etoricoxib appears to be rapid with the t_{\max} occurring approximately within 1 hour (either 1 ± 0.51 hr, 0.82 ± 0.11 hr or 0.79 ± 0.1 hr for 0.5, 1 or 2 mg/kg b wt dose) after oral administration. In their study, Dallob et al., (2003) reported a median time of about 1 hour for the occurrence of C_{\max} and t_{\max} following the characterization of etoricoxib in humans. In the present study, the $t_{1/2}$ was shown to be 10.61 ± 3.19 hr, 8.30 ± 1.44 hr and 11.51 ± 1.56 hr for the three different concentrations administered to race horses. The slight difference in $t_{1/2}$ seen in the present study is probably due to the dose of etoricoxib and more so the animal species.

In a previous study by Agrawal et al., (2001) to investigate the dose proportionality, have reported a mean $t_{1/2}$ value of approximately 22 hours. In another related study on the pharmacokinetics of etoricoxib in patients with hepatic impairments by Agrawal et al., (2003b) reported a $t_{1/2}$ of approximately 27.3 hours. Similarly, a C_{\max} of 3.1 $\mu\text{g/ml}$, t_{\max} of 0.5 hours and a terminal half life of 25.4 hours have been reported in rats for a single oral dose of 15 mg/kg b wt of etoricoxib (Radwan *et al.*, 2009). Interestingly, the terminal half life of etoricoxib was around 11.51 ± 1.56 hr for the highest dose of 2 mg/kg b wt employed in this study. This is a markedly higher and a different value compared to those reported in humans. This discrepancy may be due to large size of the liver and heavy hepatic blood flow in horses compared to humans. The concentration of celecoxib and etoricoxib along with their metabolites in blood, urine and faeces is shown in table 4.5.

Following oral administration, the peak plasma concentration of etoricoxib occurred within 1 hour after administration and the unchanged drug was detected for up to 72 hours following oral administration. The plasma samples also showed 6'-hydroxy

Table 4.5: Concentration of either celecoxib or etoricoxib (mean \pm sd, ng/ml) and their metabolites in blood, urine and faeces.

		BLOOD	URINE	FAECES
CELECOXIB		1157.87 \pm 323.58	4.8 \pm 1.44	287.77 \pm 53.84
COOH-CELECOXIB		927.97 \pm 103.21	105.53 \pm 19.09	91.12 \pm 40.22
OH-CELECOXIB		traces	\approx 3	---
ETORICOXIB	0.5 mg/kg	383.72 \pm 72.93	51.22 \pm 16	\approx 3
	1 mg/kg	512.46 \pm 67.81		
	2 mg/kg	975.84 \pm 97.98		
6'-HYDROXYMETHYL ETORICOXIB		364.31 \pm 36.48	310.13 \pm 48.11	78.20 \pm 6.56
6'-COOH ETORICOXIB		---	149.24.05	32.69 \pm 6.53
6'-HYDROXYMETHYL-1-N-OXIDE ETORICOXIB		---	37.64 \pm 57	traces

methyl etoricoxib as the possible metabolite which could be detected for nearly 96 hours. Etoricoxib in urine was in low levels. Mass spectral analysis of the unhydrolysed urine demonstrated the presence of 6'-carboxy etoricoxib together with 6'-hydroxymethyl etoricoxib as a large hump and 6'-hydroxymethyl etoricoxib-1'-N-oxide. This large hump was found to have two peaks co-eluting together with masses m/z 375 and 551. Following the hydrolysis of urine samples, the results have shown the presence of 6'-hydroxymethyl etoricoxib as the major metabolite with no traces of m/z 551. This observation has indicated that the m/z 551 was 176 Da units more than m/z 375 suggesting a glucuronic acid fraction of 6'-hydroxymethyl etoricoxib. The 6'-carboxy etoricoxib was the major metabolite in humans (Rodrigues *et al.*, 2003).

Study of the metabolism of etoricoxib in urine and faeces of horses in this study showed that 6'-hydroxymethyl etoricoxib and not 6'-carboxy etoricoxib is the major metabolite. The hydroxylation of the 6'-methyl moiety was more predominant than the 1'-N-oxidation. This is in full agreement the finding which was previously reported in an vitro study (Chauret *et al.*, 2001). Like celecoxib, etoricoxib is metabolized via methyl hydroxylation and further oxidized to form its corresponding carboxylic acid (Paulson *et al.*, 2001). Unlike etoricoxib, celecoxib is excreted as a large fraction in faeces (Paulson *et al.*, 2000a).

In conclusion, the results of this study have clearly shown that both celecoxib and etoricoxib as either parent drugs or either of their hydroxylated or carboxylated metabolites can be detected in plasma, urine and faecal samples of horses following oral administration. The parent celecoxib was identified readily in post administration plasma samples, peaking at about 1 $\mu\text{g/ml}$, 4 hours after dosing and declining thereafter. The concentration of the parent celecoxib in urine was very low (<5 ng/ml) when

compared to plasma, suggesting extensive metabolism. The major and minor metabolites were carboxycelecoxib and hydroxyl-methylcelecoxib with no evidence for the presence of glucuronidated metabolites of celecoxib. Similarly, etoricoxib levels in either plasma, faecal or urine samples show more or less the same pattern of metabolism compared to celecoxib. Together, the present results have validated the metabolism of the two coxibs in horses over time. These results may be of paramount importance for the horse racing industry especially during the administration of the two drugs as analgesics to horses before a race in order to reduce pain. Traces of the drugs and metabolites can be detected in small quantities for almost 4-5 days following oral administration.

CHAPTER - 5

METABOLIC PROFILLING OF CELECOXIB AND ETORICOXIB USING ISOLATED LIVER MICROSOMES AND TISSUES

5.1. Introduction

Real life drug metabolism studies are usually conducted on live animals *in vivo* in order to provide important results of all possible transformations of the drug over time. However, for ethical reasons, it is not possible to study the *in vivo* metabolism in most cases especially if the drug can induce adverse side effects. An elucidation of metabolism is an important part of any ADME studies since it can increase knowledge and moreover, enhance understanding of the metabolic pathway(s) of the drug. The process also helps to develop analytical methods for drug screening in complex matrices such as urine, plasma and stool samples. Screening of unknown biological samples following a race is very challenging and thus, *in vivo* and *in vitro* metabolic studies provide vital information especially if the parent drug is either quickly eliminated or completely metabolized by the racing animal. Typically, standards can be purchased, but in most instances, pure reference standards for possible metabolites are not commercially available and moreover, the synthesis of most metabolites in a laboratory is extremely laborious.

The liver is the most important organ in the body which is involved in the biotransformation of xenobiotics and within this main organizational unit, the microsomes are commonly used to understand the phase I metabolism of drugs. The metabolites formed can be tentatively identified by tandem mass spectrometry fragmentation pattern. These metabolites produced by microsomes can be used as reference compounds to identify similar compounds in biological samples. The preparation of liver microsomes is a cumbersome process and moreover, it is very expensive if procured commercially. Fresh liver has to be cut into small pieces, homogenized and centrifuged at high speeds using ultra centrifuge which would not be

available in most laboratories. The use of fresh liver slices or fragments, instead of microsomes, is a cheap option to employ in performing the same *in vitro* experiments. Thus, in this study an attempt was also made to use camel liver tissues, which can be employed as an alternative to microsomes, less expensive and moreover, less time consuming compared to the harvesting of microsomes from liver.

In order to corroborate the metabolic profiles of celecoxib and etoricoxib in horses *in vivo*, the present study employed horse liver microsomes and fresh camel liver fragments to investigate the time course metabolism of the two coxibs *in vitro* for comparison.

5.2 Method

As described in chapter-2

5.3. Result

5.3.1 Metabolism of etoricoxib by horse liver microsomes

The metabolic profile of etoricoxib in *in-vitro* by horse liver microsomes supplemented with NADPH was done employing LC/MS, similar to that described earlier in chapter-4. The results show the formation of one metabolite which was confirmed by LC/MS to be 6'-hydroxy methyl etoricoxib and with a mass to charge of 375 Da. The CID pattern of this metabolite formed in the presence of NADPH had fragments of m/z 357, 339, 330, 296 and 278, similar to those shown earlier in Figure 4.17 for etoricoxib. Based on the LC/MS analysis performed on a TSQ Quantum ACCESS in the positive ion mode, the mass ($M+H$) ion was shown to be 16 Da which was higher than etoricoxib (m/z 375). This interesting finding has indicated that the metabolite formed is more or less similar to the metabolite detected in urine in the horses *in vivo*. The results also show that in the

absence of NADPH, the incubation mixture containing etoricoxib and liver microsomes failed to produce any detectable trace of the metabolite when screened by LC/MS, indicating that the metabolite formation was enzymatic and NADPH dependent. The present results also reveal that a selected ion monitoring for m/z 375 displayed a single peak at the retention time, similar to that of the urinary metabolite 6'-hydroxy methyl metabolite obtained in the *in vivo* studies for the horses. The presence of etoricoxib-1'-N oxide with m/z 375 (exact mass as 6'-OH-methyl etoricoxib) was not confirmed even with selected ion monitoring (SIM) run on an LC/MS. Thus, the absence of etoricoxib-1'-N oxide *in vitro* confirms that the metabolite is not present even in urine, after the administration of etoricoxib *in vivo*. Therefore, the corresponding glucuronide conjugate of m/z 551 detected in urine matrix has probably derived from the conjugation of glucuronic acid to the 6'-hydroxy methyl etoricoxib. The results presented in Figure 5.1 also show no evidence for the presence of 6'-carboxy etoricoxib and 6'-hydroxymethyl-etoricoxib-1'-N-oxide. In contrast, both 6'-carboxy etoricoxib and 6'-hydroxymethyl-etoricoxib-1'-N-oxide were obtained as metabolites in urine and faeces samples from horses.

5.3.2. Metabolism of celecoxib by horse liver microsomes

In this series of experiments, LC/MS/MS technique was also used to measure the two metabolites derived from celecoxib following its incubation with horse liver microsomes in the presence and absence of NADPH over a period of 0 minute (control) to 180 minutes. The qualitative results are presented in Figure 5.2 and they show the (A) the presence of celecoxib in the microsomal mixture and (B and C) the complete absence of characteristic peaks for hydroxyl celecoxib and carboxy celecoxib, respectively.

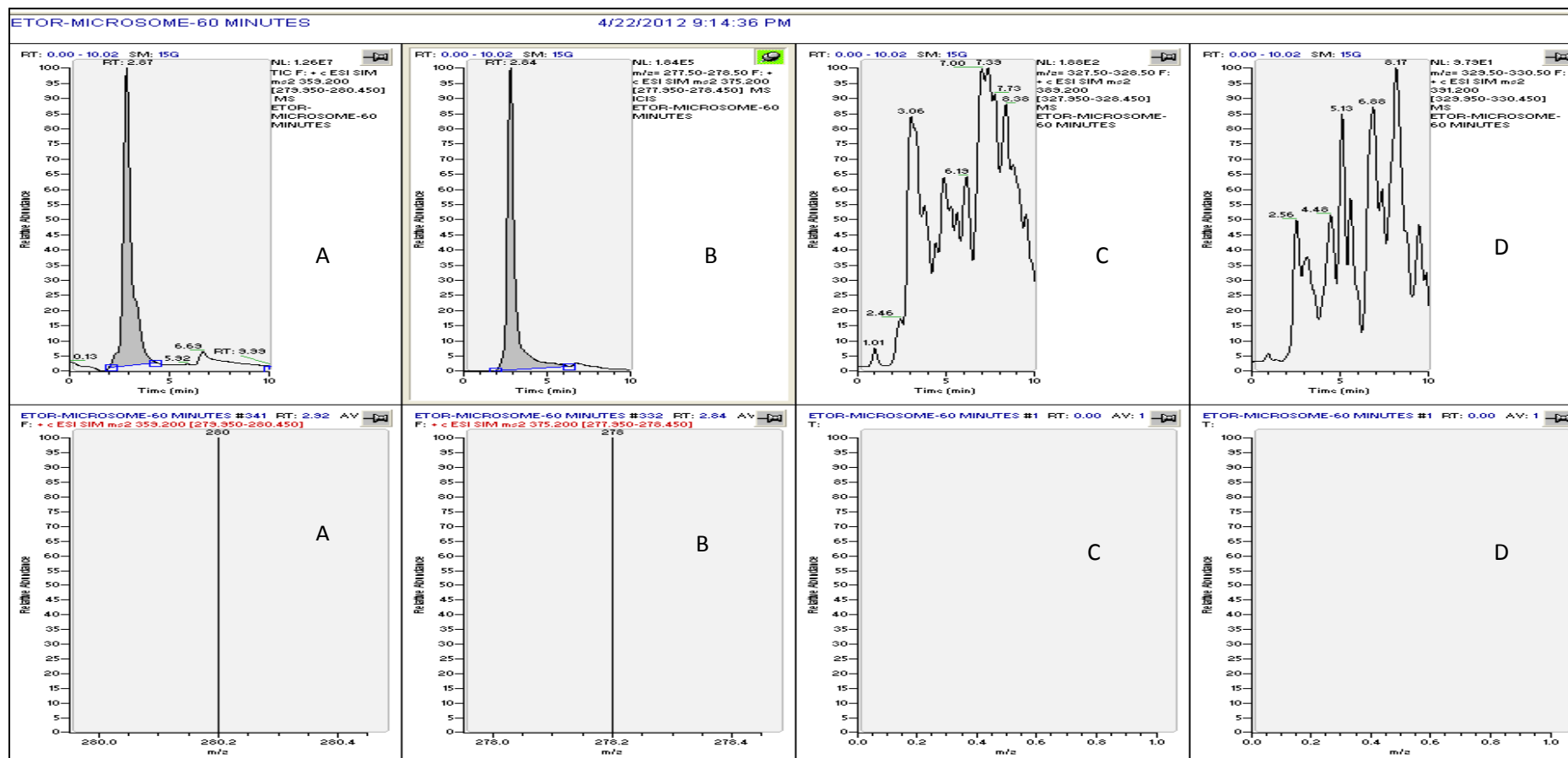


Figure 5.1: Original chart recordings showing the LC/MS/MS chromatogram (above) and SIM fragment ion (below) of (A) etoricoxib (m/z 280), (B) 6'-hydroxymethyl etoricoxib (m/z 278), (C) 6'-carboxy etoricoxib (m/z 328) and (D) 6'-hydroxymethyl-etoricoxib-1-N-oxide (m/z 330) obtained after etoricoxib incubation with horse liver microsomes, n=3.

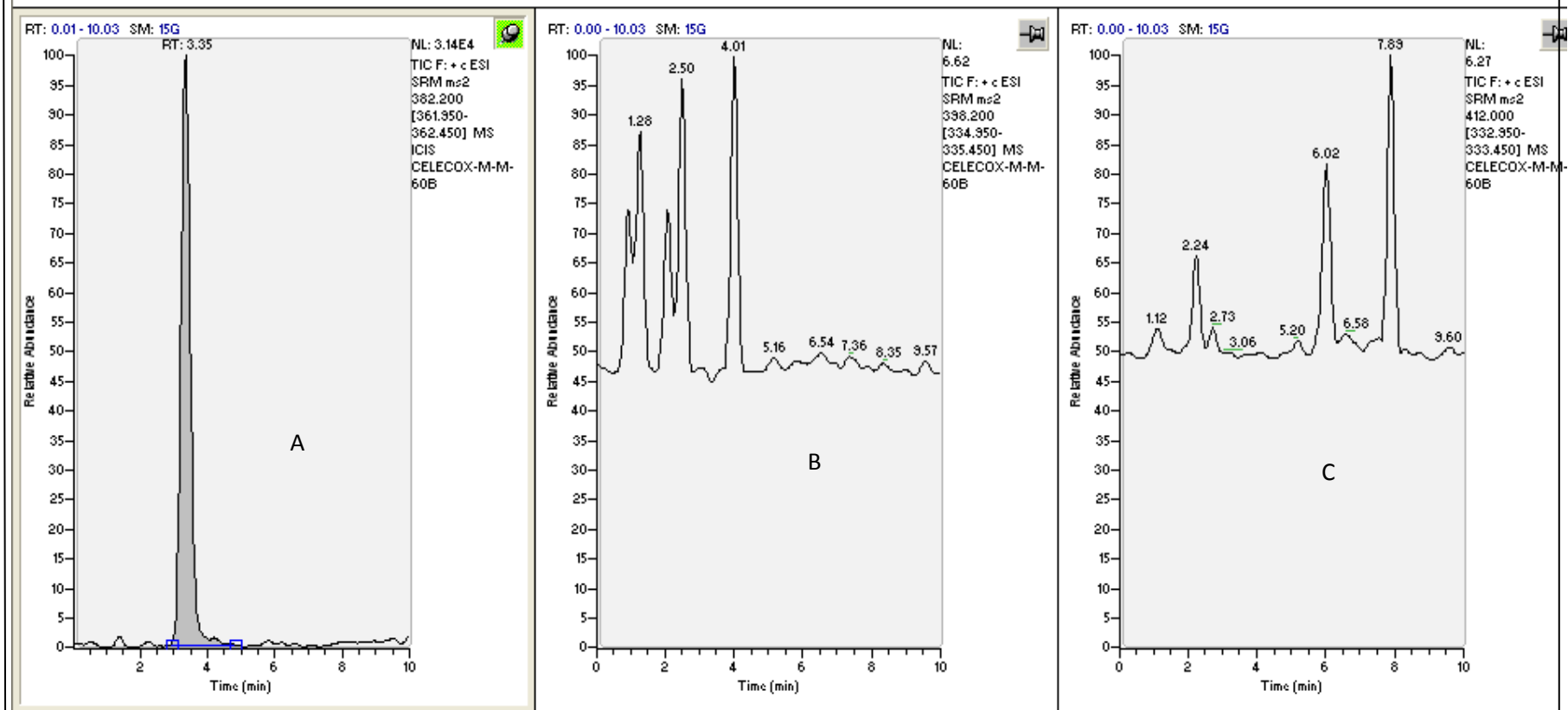


Figure 5.2: Original chart recording showing the chromatograms and SRM fragment ion of (A) celecoxib, (B) the absence of hydroxy-celecoxib shown as no characteristic peak and (C) the absence of carboxy celecoxib shown as no characteristic peaks in horse liver microsomal incubation; n=3.

5.3.3 Metabolism of etoricoxib and celecoxib by camel liver tissue.

The procedure for the *in vitro* metabolic study using camel liver tissue is same as that used for horse liver microsomes. Either etoricoxib or celecoxib was separately incubated with camel liver slices of 0.25 – 0.5 cm cubes or fragments in the presence and absence of NADPH from time 0 minute (control) to 180 minutes.

Following incubation of etoricoxib with fresh camel liver slices, there was no metabolite detected in the control sample which had no NADPH. Figure 5.3 shows original chart recordings of chromatograms and corresponding SIM fragment ions following incubation of etoricoxib with camel liver slices in the presence of NADPH. The results show etoricoxib can be metabolized by the liver tissues to produce the metabolite, namely 6'-hydroxymethyl etoricoxib within 30 minutes following incubation.

In addition, the data further reveal that after 60 minutes of incubation in the presence of NADPH, etoricoxib together with its metabolites, 6'-hydroxymethyl etoricoxib, 6'-carboxy etoricoxib and 6'-hydroxymethyl etoricoxib-1'-N-oxide were also detected in the incubation medium. This confirmation was based on their masses and fragmentation pattern on LC/MS/MS. Similarly, Figure 5.4 shows the LC/MS/MS chromatograms and the corresponding SIM mass fragmentation of celecoxib and its metabolites following incubation with camel liver tissue *in vitro*.

The results show that hydroxy celecoxib and carboxy celecoxib could be detected in incubation medium and the parent celecoxib is converted into its metabolites within 60 minutes of incubation with NADPH. It is particularly note worthy that both hydroxy celecoxib and carboxy celecoxib are the two metabolites produced *in vivo* after celecoxib

administration to horses. Table 5.1 show the comparison of metabolites formed from the two COX-2 inhibitors in vivo and in vitro. It has been noted that 6'-hydroxymethyl etoricoxib was obtained during all the treated conditions.

Carboxy celecoxib was obtained from all conditions except when celecoxib was incubated with liver microsomes. In contrast, hydroxy celecoxib and glucuronide of 6'-hydroxymethyl etoricoxib were only produced in urine samples. In addition, 6'-carboxy etoricoxib and 6'-hydroxymethyl-1'-N –oxide of etoricoxib were obtained only in urine and faeces and when incubated in camel liver tissues.

Together, the present in vitro experiments employing camel liver tissue corroborate the results obtained from in vivo studies in horses (see table 5.1).

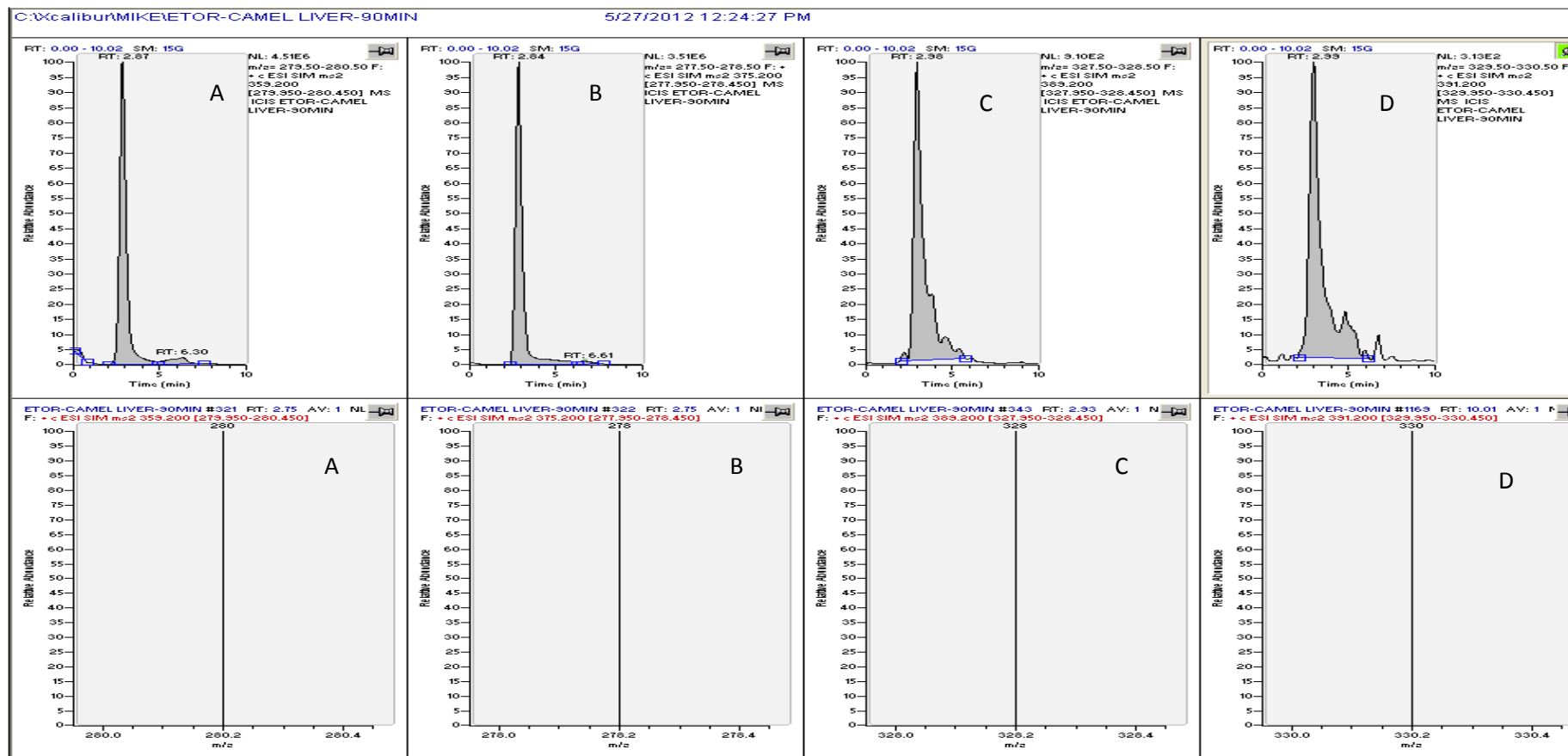


Figure 5.3: Original chart recordings of chromatograms and corresponding SIM fragment ions of (A) etoricoxib, (B) 6'-hydroxymethyl etoricoxib, (C) 6'-carboxy etoricoxib and (D) 6'-hydroxymethyl-etoricoxib-1'-N-oxide identified from incubation of etoricoxib with camel liver tissues, n=3.

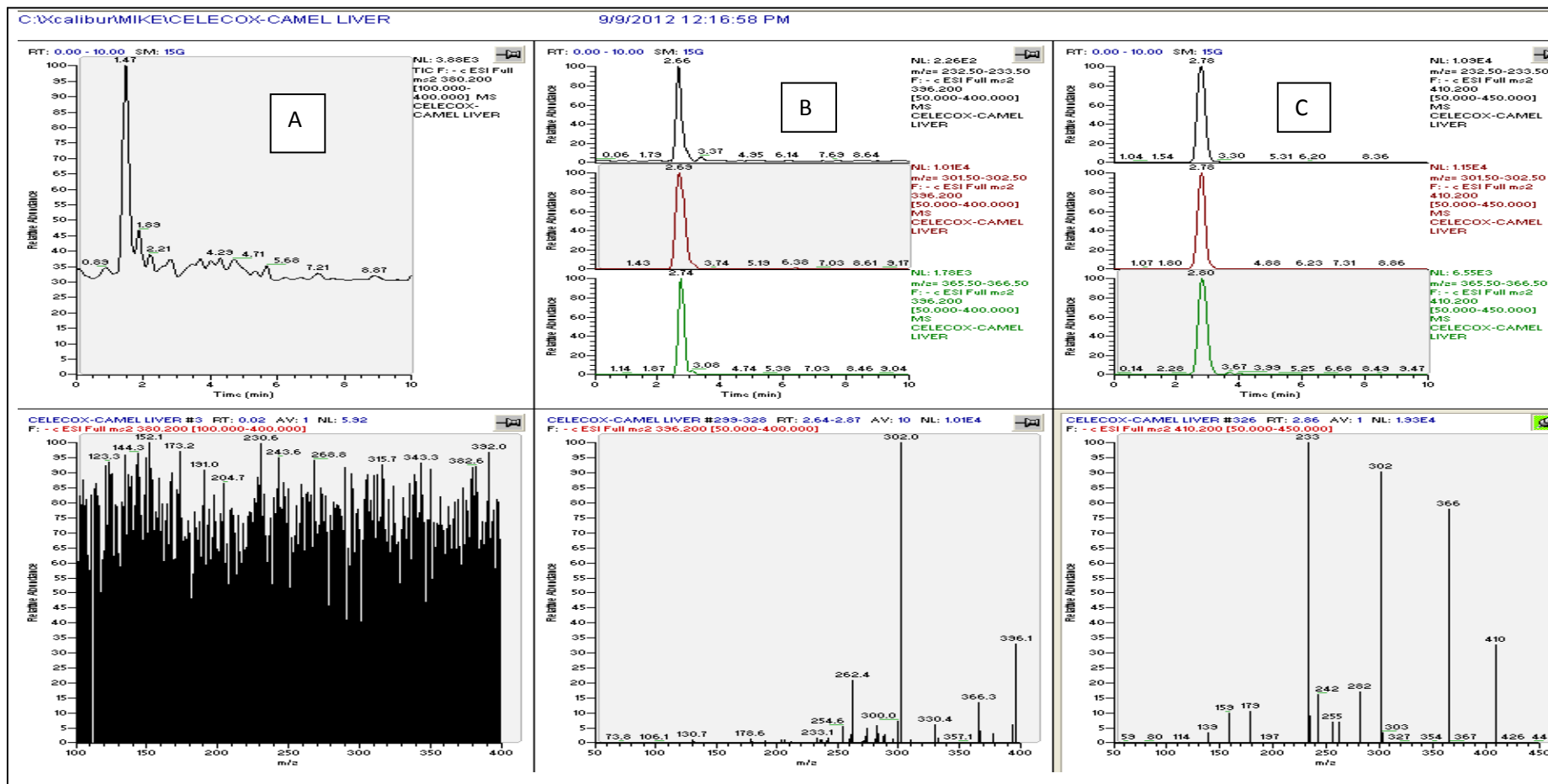


Figure 5.4: Original chart recordings of camel liver tissue *in vitro* metabolism of celecoxib showing (A) chromatographic peak for celecoxib, (B) chromatographic peak for hydroxy celecoxib and (C) characteristic chromatographic peak for carboxy celecoxib, n=3.

Table 5.1: Comparison of metabolites formed *in vivo* and *in vitro*

Metabolites	<i>In vivo</i>			<i>In vitro</i>	
	Plasma	Urine	Faeces	Using microsomes	Using camel liver
<u>Celecoxib</u>					
Hydroxy-celecoxib	Tr	√	N/A	N/A	√
Carboxy-celecoxib	√	√	√	N/A	√
<u>Etoricoxib</u>					
6'-hydroxymethyl-etoricoxib	√	√	√	√	√
6'-carboxy-etoricoxib	N/A	√	√	N/A	√
6'-hydroxymethyl-etoricoxib-1'-N-oxide	N/A	√	√	N/A	√
Glucuronide of 6'-hydroxymethyl-etoricoxib	N/A	√	N/A	N/A	N/A

Tr- traces detected, N/A-not detected, √ - detected

5.4. Discussion

The study of drug metabolism using *in vitro* methods has become widespread in the field of drug discovery and it is also being used to a lesser extent in sport drug surveillance if live animals are not available for experiments involving the administration of the drug *in vivo*. Several previous studies have been published investigating the phase I metabolism of anabolic steroids such as turinabol, mesterolone and non-steroidal anti-inflammatory drugs including phenylbutazone etc.(Ho *et al.*, 2007; Leung *et al.*, 2005). The metabolic profile of a drug obtained *in vitro* usually reflects the metabolic pattern *in vivo* and thus in drug discovery stages greater importance is given to predict the safety and efficacy of the drug (Wrighton *et al.*, 1993). More recently, two equine cytochrome P450 enzymes, namely CYP2D50 and CYP2C92 have been sequenced (DiMaio Knych & Stanley, 2008). Though the equine and human isoforms share some substrate specificity, they nevertheless differ in their enzyme kinetics and the metabolites they produce (Knych *et al.*, 2010). In a recent study, Peters *et al.*, (2013) annotated and cloned the CYP2B6 enzyme in horses and they studied the phase I metabolism of ketamine, a drug regularly administered to calm down horses during long travel and they found that the enzyme produced norketamine and hydroxylated metabolites of norketamine. Again, DiMaio-Knych *et al.*, (2011) studied the metabolism of dantrolene, a muscle relaxant to characterize the cytochrome P450 enzyme involved and reported that dantrolene was rapidly metabolized to 5-hydroxy dantrolene both *in vivo* and *in vitro*. They further reported that two enzymes were responsible for the metabolism of dantrolene, as evidenced by two distinct K_m values.

The aim of the present *in vitro* study employing horse liver microsomes and fresh camel liver fragments was primarily to identify and to corroborate the metabolites formed *in vitro* compared to those formed *in vivo* in horses for the two COX-2 inhibitors, etoricoxib and celecoxib normally used in humans and in other animals to relieve pain and inflammation. It is the belief that an understanding of the metabolism and elimination time of the drugs and their metabolites would help in increasing the coverage of any miss-usage of these analgesics when they are prescribed to treat pain and inflammation in racing animals like horses. Thus, the molecular structure or the microsome stability parameters are not fully rationalized beyond the assignment of the molecular masses of the metabolites detected in a biological sample.

Normally, a forensic dope testing laboratory can detect a substance mainly on its molecular mass and fragmentation pattern of the analyte employing a particular analytical equipment. A biological sample is declared positive on a comparison between the sample in question and the reference (drug/metabolite). In most instances, the purified standards for most metabolites are not commercially available for comparison. Hence, the metabolites formed by *in vitro* experiments using liver microsomes or fresh liver slices are allowed to be used as reference materials (Wong *et al.*, 2011). Horses are not slaughtered in the UAE for human consumption and procurement of horse liver for *in- vitro* studies from the local abattoir is highly impossible, while camels are slaughtered and camel meat is a delicacy in Arabic cuisine. Hence camel liver tissues were used in this part of the study. Thus, the non availability of fresh horse liver tissues prompted this study to use fresh camel liver tissues instead, to study the metabolism of etoricoxib and celecoxib.

Organic solvents like methanol, acetonitrile or DMSO are often used in *in-vitro* experiments if the drug in study is poorly soluble in either water or buffers (Chauret *et al.*, 1998). The two new COXIB drugs, etoricoxib and celecoxib have different solubility properties. Etoricoxib is easily soluble in water which was used to dissolve the drug in this *in-vitro* experiment. On the other hand celecoxib was not soluble in water and other organic solvents like methanol or ethanol. However, celecoxib was found to be readily soluble in either acetonitrile or DMSO.

The present *in-vitro* experiments were performed with celecoxib dissolved in DMSO, which is less than 1% of DMSO used in the entire *in-vitro* incubation experiment. DMSO is a prescription medicine, mostly used as a topical application for pain and inflammation (Nikonova, V, 1998). Cytochrome P450 are the principal family of enzymes employed in drug metabolism and CYP3A4 is the major enzyme in the liver to metabolise drugs. The lipophilic nature of few new drug candidates often require organic solvents such as acetonitrile and dimethyl sulfoxide for their solubilization to perform *in vitro* incubation experiments. These organic solvents have been found to have either inhibitory or stimulatory effects on the enzymes involved in drug metabolism (Busby *et al.*, 1999).

Nishiya *et al.*, (2010) studied the effect of DMSO in the metabolism of diazepam and concluded that DMSO competitively inhibits the formation of diazepam metabolites *in vitro*. The toxic effects of DMSO on gene expression in human and rat haepatocytes have demonstrated revealing that the organic solvent was non-cytotoxic up to a concentration of 2% (v/v) (Sumida *et al.*, 2011). Iwase *et al.*, (2006) evaluated the effect of solvents like acetonitrile, methanol, ethanol and dimethyl sulfoxide on the inhibition

of testosterone and midazolam using pooled human liver microsomes. They concluded that all organic solvents above 1% had an inhibitory effect on a substrate concentration dependant or on a time-dependant manner except for methanol which had no inhibitory effect on *in vitro* metabolism studies. Another *in vitro* study of the effect of DMSO on dextromethorphan and phenacetin using human liver microsomes suggests that DMSO should be used at less than 1% (w/v) concentration and a concentration of 5% strongly could affect the drug metabolism *in vitro* (Chauret *et al.*, 1998).

The present initial developmental studies showed that DMSO had no detectable effect on the metabolism of celecoxib in the presence of fresh camel liver tissues by the formation of characteristic metabolites of celecoxib as shown in figure 5.4. The control experiments in this study were performed either in the presence or in the absence of either horse liver microsomes or camel liver slices or NADPH. Thus, the control experiments did not form any of the corresponding metabolites.

5.4.1. Metabolism Etoricoxib

Incubation of etoricoxib in the presence of human liver microsomes has previously been reported to give one major metabolite, namely 6'-hydroxymethyl etoricoxib and one minor metabolite, etoricoxib-1-N-oxide, both having a m/z 375Da and seen as two peaks when analyzed by HPLC/APCI-MS (Chauret *et al.*, 2001). In addition to the two metabolites, a third metabolite of m/z 389 has been reported using human haepatocytes (Nicoll-Griffith *et al.*, 1999). In the present study, the use of horse liver microsome yielded only one metabolite as seen as a single peak of m/z 375 throughout the entire LC/MS analysis. Oral administration of etoricoxib to horses has also shown previously to have a single peak for m/z 375 as mentioned in chapter 4 of this study. This m/z 375 further forms a glucuronic acid metabolite *in vivo*. Therefore, the metabolite noticed in

in vitro could be possible the 6'-hydroxy methyl etoricoxib. The absence of etoricoxib-1-N-oxide in *in vitro* microsomal incubation and *in vivo* experiments suggest that horses primarily metabolize etoricoxib to 6'-hydroxymethyl etoricoxib only and this metabolite is further broken down to other metabolites. Kassahun *et al.*, (2001) reported that the disappearance rate of etoricoxib during the formation of 6'-hydroxymethyl etoricoxib was 4.2 ± 2.7 hr (mean \pm S.D; n=3) showing low intrinsic clearance *in vitro*. The present *in vitro* metabolic stability study of etoricoxib with horse liver microsome shows a high *in vitro* clearance rate. In a previous study, (Eddershaw & Dickins, 1999) employed a metabolic stability assay to compare *in vitro* experiments with *in vivo* studies to investigate drug metabolism and they showed that the metabolism of the drug *in vitro* was a reasonable estimate compared to the *in vivo* half life of the drug. The results presented in this study show that the terminal half life for either coxib drug estimated in the microsomal study correlates closely to the *in vivo* data shown in table 4.4 of this study (Chapter 4).

5.4.2. Metabolism of Celecoxib

Celecoxib has been reported to form hydroxy celecoxib by human liver microsomes (Iyer *et al.*, 2004). Similarly, Tang *et al* (2000) have reported that celecoxib forms only hydroxy celecoxib with no trace of carboxy celecoxib. However, in the present study with horse liver microsomes, the characteristic hydroxy celecoxib and carboxy celecoxib were not detected when screened by LC/MS/MS analysis. In contrast, when celecoxib was incubated with camel liver tissue it produced both hydroxy and carboxy metabolites of celecoxib.

A correlation between *in vitro* and *in vivo* data are often used in the pharmaceutical industry to reduce drug development time and optimise formulation (Scheubel

Emmanuel, 2010). This *in vitro* study employing both horse liver microsomes and fresh camel liver slices has shed further light into the metabolism of these COX-2 inhibitors compared to the *in vivo* whole animal situations, employing horses. These *in vitro* experiments closely corroborate the findings obtained in the *in vivo* studies for both coxib drugs (see table 5.1 for comparison). It is apparent that liver microsomes, the liver fragments and the whole animal can all metabolize the drug, at least etoricoxib, whereas, in the majority of cases the liver and the whole animal are required to metabolize both drugs fully.

From the present study it is true to say that either celecoxib or etoricoxib can be administered to the six horses to alleviate pain. The animals can metabolise the drugs *in vivo* to produce a number of metabolites over time. Moreover, *in vitro* NADPH-dependent metabolism of the two COX-2 inhibitors employing native horse liver microsomes can also help to identify and to determine the different metabolites produced by the parent drugs in their purest form rather than finding them in low abundance in such dirty matrices as urine, plasma or faeces which are normally contaminated with numerous biological substances.

Kassahun *et al.*, (2001) studied the metabolic profile of etoricoxib and they found it to be less complex and moreover, it involved the formation of P450-dependent 6'-methyl hydroxylation and 1'-Noxidation as the clearance pathway, the latter being the major metabolite in human liver microsomes. This metabolite is oxidized further to 6'-carboxy etoricoxib in the presence of co-factor-fortified human liver cytosol. Unlike microsomes, 6'-carboxy etoricoxib has been identified as the major metabolite following oral or intravenous administration in humans (Rodrigues *et al.*, 2003).

Tang et al., (2000) report that methyl hydroxylation is a major pathway of celecoxib metabolism using human liver microsomes and the reaction is catalyzed mainly by CYP2C9 enzymes and CYP3A4 seems to play a minor role in the metabolism of the parent drug. It has been reported that the action of CYP2C9 enzyme is greater than CYP3A4 in the whole liver compared to liver microsomes (Tang *et al.*, 2001). Thus, in the present study, the CYP2C9 enzyme present in camel liver slices could probably have metabolized celecoxib more effectively than horse liver microsomes.

Similar to etoricoxib, the metabolism of celecoxib is also relatively simple. The parent celecoxib is metabolised to hydroxyl celecoxib which is oxidized further to the corresponding carboxylic acid metabolite via cytosolic alcohol dehydrogenase (Sandberg *et al.*, 2002).

In conclusion, the results of this study have clearly shown that isolated liver tissues from both camel and horse can metabolize both celecoxib and etoricoxib almost similar to in vivo studies employing horses only. Both horses and camels are routinely used for racing at least in the UAE and any misuse of these drugs to relieve pain and inflammation in the animals prior to a race may also help to enhance their performance. In the racing fraternity, this can be interpreted as an illicit use of the drug. Thus, caution has to be taken into consideration when prescribing these coxib drugs to relieve pain on these racing animals prior to a race.

CHAPTER – 6

GENERAL DISCUSSION

6.1. Introduction

The horse has played an important role in the development of human civilization helping mainly in transportation and to win wars and moreover, winning money as in horse racing. The latter has now been described as a royal sport with huge prize money in the offering. A variety of sporting activities take place in United Arab Emirates (UAE) and the richest racing sport in the world, the Dubai World Cup is held in Dubai annually, with a purse of six million United States dollars. Racing is held throughout the world in countries like the United Kingdom, the United States of America, Australia, UAE and in many other countries. The huge prize money has led to many vice means of winning the race, which has led to strict regulations to be adopted by the racing fraternity in order to ensure that the sport is clean of illicit use of drugs when treating the horse for pain relief. This usually occurs before a race since it can enhance the performance of the horse in endurance racing. In the sporting fraternity, this illicit use of a drug to enhance the performance of the horse is referred as doping.

Anabolic steroids and analgesics are widely used in the treatment of sickness and pain in veterinary animals and moreover, analgesics, like aspirin, have been used to reduce pain and inflammation (Kumar & Clark, 2009; Vane & Botting, 2003). During this process, they inhibit the COX-1 and COX-2 enzymes thereby leading to unwanted side effects like ulceration of the stomach lining and gastrointestinal bleeding (Vane & Botting, 1998a). Recently, a new generation of NSAIDs have been developed by the Pharmaceutical Companies and they are called COX-2 inhibitors (Marnett, 2009). These new analgesics have been found to be more effective in reducing pain compared to others, like aspirin, without inhibiting COX-1, thereby decreasing any harmful side

effects. Celecoxib and etoricoxib are two such new drugs which are used routinely in human medicine for the treatment of osteoarthritis and rheumatoid arthritis as well as pain relief and inflammation (Paulson et al, 2000a; Rodrigues et al, 2003; Marnett, 2009). Since these two coxibs are effective pain killers in humans, they could also be used to treat large veterinary animals, including camel and horses. Prior to the study, no such other investigation was done with these two coxibs in either horses or camels.

As such, this project was specifically designed to investigate the therapeutic effects of celecoxib and etoricoxib in six retired race horses measuring a number of haematological and clinical biochemical parameters as well as determining the metabolism and elimination (pharmacokinetics) of each drug by the animals. In tackling the scientific problems, blood, urine and faeces were collected for analysis using well established biochemical and chemical techniques. In order to corroborate the metabolism of the two drugs *in vivo*, an *in vitro* study was also done employing commercial horse liver microsomes and fresh isolated camel liver to metabolize the drugs over time. The results are presented in chapters 3-5 of the thesis. An attempt will now be made to critically compare the present data with those previously reported in the literature, highlighting the clinical parameters, side effects, pharmacokinetics of the drugs and metabolism by microsomes and camel liver tissues.

6.2. Measurements of haematological parameters

The data presented in the present study have clearly demonstrated that oral administration of physiological doses of either celecoxib or etoricoxib to the six race horses had neither no adverse side effects to the animals nor any significant effect on blood clinical parameters compared to data obtained prior to the administration of each

drug to the animals, except for a small transient elevation of creatinine with etoricoxib. In a previous study, Koene *et al.* (2010) investigated the haematological parameters like RBC, WBC, platelets, Hb, MCV.MCH, basophils, neutrophils, lymphocytes, monocytes, eosinophils, together with liver and kidney function enzymes in horses after oral administration of a therapeutic dose of firocoxib (a new COX-2 inhibitor developed specifically for veterinary animals).

Like the present study, these authors found that all these clinical parameters were within the normal reference range and none were considered clinically significant. Firocoxib is part of the coxib family, but with a completely different chemical structure compared to either celecoxib or etoricoxib and with a molecular weight of 336.4 (see Figure 1.7 for comparison; Marnett, 2009). Similarly, Emery *et al.*, (1999) reported that human patients receiving 200 mg of celecoxib twice daily for 24-weeks show no change in the liver-function enzyme levels. In contrast, Kockaya *et al* (2010) studied the effect of high pharmacological doses of celecoxib in rats employing concentrations of 10 mg/kg and 50 mg/kg/day over 28 days. They showed that these large doses of celecoxib can significantly elevate plasma levels of AST and GGT, the two enzymes which are mainly found in the liver. An elevation of these two enzymes in the blood is an indication that they can alter the liver leading to either damage or obstruction (Kumar and Clark, 2009).

In the present study, it has been noticed that celecoxib administration at a concentration of 2 mg/kg b wt did not alter the levels of the liver enzymes, namely AST, ALT and GGT indicating that the horses can tolerate this therapeutic dose without causing any liver dysfunction. It would be interesting to increase the dose of the coxib gradually in order to determine the maximal dose which the animals can tolerate. In this respect, it

may take a longer time for the animal to eliminate the drug compared to the five days obtained in this study. The rationale for the present study was to use a therapeutic dose rather than a pharmacological dose taking ethical issues and the welfare of the animals into consideration. Moreover, since this was a forensic study in part, it was relevant to employ low doses in order to determine the elimination time of both the parents drugs as well as the metabolites as some of these NSAIDs are routine used in the racing industry.

It is also noteworthy that in addition to their analgesic effect and gastrointestinal friendliness, COX-2 inhibitors are seen to lower lipid levels in humans. In a study, Chow *et al.*, (2005) treated breast cancer patients with celecoxib and they found that the drug was able to reduce their total cholesterol and lipids. The presented study did not measure either LDL, HDL or triglyceride levels. This may be an area for future investigation at least in the horse.

Baracho *et al* (2009) have reported that chronic administration of etoricoxib at 30 mg/kg/day to rats produced a significant increase in the levels of red blood cells, haematocrit and platelets compared to control untreated groups. Furthermore, they reported that etoricoxib administration did not produce significant alteration in other haematological parameters. Behal *et al* (2009) reported an overall decrease in total leucocyte, neutrophils, lymphocytes, monocytes and eosinophils when rats were treated with 0.64 mg/kg bwt and a significant increase in the leukocyte count when rats were administered ten times the earlier dose. Histological sections of colon and kidney did not produce major histo-architectural changes with etoricoxib administration.

The present study investigated all haematological parameters after administration of celecoxib and etoricoxib and found no alteration in the blood- borne clinical parameters including the anions and cations, except for a slightly elevated level of creatinine in the horses at about 4 hours after oral administration of etoricoxib. This elevated level of creatinine is by far within the acceptable reference values in horses. The level was found to decline back to normal values within 24 hours. An elevated plasma creatinine level is an indication of kidney damage.

6.3. Some adverse side effects of NSAIDS

Traditional NSAIDs like aspirin and related drugs which can inhibit both COX-1 and COX-2 were developed mainly to treat humans, but they have also been used to treat veterinary animals causing mucosal erosion and haemorrhage, especially in dogs and cats (Conlon, 1988). Similarly, phenylbutazone and flunixin have been shown to induce high incidence of gastric ulcer following long term usage in horses (Goodrich et al, 2006). In another study, it was demonstrated that concurrent administration of a combination of drugs including celecoxib and aspirin which can inhibit both COX- 1 and COX-2 can produce stomach ulcers in rats, compared to no gastrointestinal tract (GIT) damage when celecoxib was administered separately (Rahme *et al.*, 2002). Because of these adverse side effects, the drugs are now coated making them slightly safer. It was previously reported that an enteric coated aspirin had less irritation to the stomach of the dog (Kauffman, 1989). Clinical studies with COX-2 inhibitors have shown that unlike traditional NSAIDs, like aspirin, COX-2 inhibitors produce either none or reduced GIT complications (Laine *et al.*, 2003; Scheiman *et al.*,2006; Laine *et al.*,2008)). The “VIGOR” study employed rofecoxib and it showed a 54% decrease in

GIT events when compared with naproxen, a traditional NSAID (Bombardier *et al.*, 2000). Likewise, Lascelles *et al.* (2005) reported that deracoxib, a COX-2 inhibitor developed for treatment of pain in dogs, produced GIT perforations when used at high pharmacological doses compared to approved or recommended dosage. In contrast, Silverstein *et al.* (2000) reported a lower incidence of ulcer complications with celecoxib at a dose of 400 mg twice daily when compared with ibuprofen and diclofenac. In dogs, a super-therapeutic dose of celecoxib did not show any GIT toxicity (Maziasz *et al.*, 1997). Similarly, Altinkaynak *et al.* (2003) reported that celecoxib did not induce damage to gastric mucosa of rats. In human studies, endoscopic examination of the stomach following treatment with celecoxib at 400 mg/day for 12 weeks showed lower incidence of gastro-duodenal ulcers (Hawkey *et al.* 2004; Goldstein *et al.* 2001). Interestingly, in the “SUCCESS-I” study, it was reported that celecoxib at either 200 mg or 400 mg/day had lower incidence of ulcer complications in patients who were treated for osteoarthritis (Singh *et al.*, 2006).

The “EDGE” study compared etoricoxib with diclofenac sodium on gastrointestinal tolerability and effectiveness and the results show that etoricoxib at 90 mg/day to humans has significantly lower GI adverse events. In contrast, diclofenac sodium increased the levels of the two liver enzymes, ALT and AST, but not with etoricoxib (Kruger, 2008). A comparative study with traditional NSAIDs like diclofenac, ibuprofen and naproxen versus etoricoxib showed that etoricoxib was well tolerated at 60 – 120 mg/day (Ramey *et al.*, 2005). A traditional NSAID study of 16 horses, comparing phenylbutazone, flunixin meglumine and ketoprofen shows that phenylbutazone had the greatest toxic potential in the form of renal necrosis compared to flunixin, while ketoprofen had the least or no side effect on the kidneys (MacAllister *et al.*, 1993). In the present study, it was shown that either celecoxib or etoricoxib

administration daily at a dose of 2 mg/kg bwt for 15 days show no ulceration or bleeding upon endoscopic examination of the stomach. Moreover, all the animals behaved normal with no symptoms of dyspepsia or diarrhoea. Together, these results clearly show that the horses can tolerate both celecoxib and etoricoxib when they are administered orally to the animals over several days.

6.4. Pharmacokinetics and Pharmacodynamics of some common NSAIDs in horses

Pharmacokinetics is concerned with the study and characterization of drug – its absorption, distribution and excretion. Additionally, it gives information of its intensity and the duration of characteristic effects (Baggot, 1995). NSAIDs are the drug of choice to alleviate pain and many studies have been established for the pharmacokinetic profiles of NSAIDs in horses. The key characteristics for moderate to good pharmacokinetics are a good bioavailability, a high degree of protein binding and a good volume of distribution (Lees *et al.*, 1988).

Veterinary and human pharmacology differ principally in the different species in which drugs are administered and studied (Lees *et al.*, 1988). It is often presumed that the dose-effect relationship are same in animals as in humans but, differences in the biochemistry and physiology among species and the different drug classes bring about variations in pharmacokinetic and pharmacokinetic activity for the same drug. It has been found that the herbivore species of animals such as the horse and other ruminants metabolize lipid-soluble drugs more rapidly than carnivorous animals. Moreover humans have been found to metabolize drugs slowly when compared to animals. These are reflected by the half-life values for certain drugs studied in humans as well as in animal species (Burns & Conney, 1964). It has been reported that the drugs such as phenylbutazone, flunixin

and meclofenamate administered through oral route tend to be absorbed on to hay and thus delay absorption. This binding leads to two peaks of plasma concentration, one from the free drug that is absorbed and the second peak from the drug released from hay digestion (Maitho *et al.*, 1986).

Lees *et al.*, (1985) studied the pharmacokinetics and bioavailability of phenylbutazone in mountain ponies. They reported that the drug clearance in young ones after intravenous administration was twice more rapid than older ponies. Moreover, after an oral administration the C_{\max} was greater in older ponies, which was due to slow plasma clearance and the 24 hour urinary excretion accounted for approximately 25% of the administered intravenous dose.

Earlier, Gerring *et al.*, (1981) administered a single oral dose of phenylbutazone (1.1 to 13.2 mg/kg) to a group of horses and report that a considerable individual variations exist in both timing and magnitude of the plasma drug response between horses. They report that phenylbutazone forms two principal metabolites, oxyphenbutazone and γ -hydroxyphenylbutazone. Furthermore, the plasma concentration of oxyphenbutazone did not exceed 25% of the parent drug and γ -hydroxy metabolite concentration never exceeded 1 $\mu\text{g/ml}$.

The plasma disposition and tolerance of the non-steroidal anti-inflammatory drug carprofen was studied in three thoroughbred horses and found to have a V_d of 0.08 to 0.32 L/kg and a plasma half-life of 14.5 to 31.4 hours with no accumulation of the drug in plasma after oral administration (Mckellar *et al.*, 1991). Similarly, Jaussaud *et al.*, (1992) reported the pharmacokinetics of tolfenamic acid after an oral administration (30 mg/kg bwt) to horses to have a peak plasma concentration of $11.1 \pm 0.69 \mu\text{g/ml}$ and

a $t_{1/2}$ of 4.2 ± 0.48 hours. They further reported that tolfenamic acid could not be detected in equine plasma beyond 48 hours after drug administration.

The pharmacokinetics and pharmacodynamics of flunixin and ketoprofen were studied by Landoni and Lees (1995) to evaluate their anti-inflammatory properties and inhibition of bradykinin-induced swelling in horses. They reported that flunixin had an elimination half-life of 3.37 ± 1.09 hours, V_d of 0.317 ± 0.126 L/kg and a clearance of 0.058 ± 0.004 L/kg/hour compared to the enantiomer ketoprofen. The R(-) and S(+) enantiomer of ketoprofen showed a $t_{1/2}$ of 1.09 ± 0.19 hour and 1.51 ± 0.45 hours, respectively. Similarly clearance was more rapid with R(-) ketoprofen than S(+) ketoprofen. Furthermore, the pharmacodynamics of flunixin and ketoprofen was studied by determining the inhibitory effects on serum thromboxane, PGE_2 and leukotriene B_4 . They established that both the drugs inhibited serum TxB_2 synthesis for up to 24 hours and flunixin was more potent in inhibiting PGE_2 than ketoprofen and neither drug had any effect on leukotriene B_4 and both the drugs inhibited bradykinin-induced swelling.

In 1994, Lees *et al.* evaluated the pharmacokinetics and pharmacodynamics of carprofen in 6 horses having nonimmune inflammation following intravenous administration of 0.7 mg/kg bwt of racemic mixture of carprofen. The pharmacokinetic parameters were 18.1 hours for $t_{1/2}$, 0.25 L/kg for V_d and 58.9 ml/min for clearance. The pharmacodynamic effect showed reduction in swelling at the site of inflammation with moderate suppression of serum thromboxane B_2 and prostaglandin E_2 synthesis.

Villa *et al.*, (2007) investigated the COX-1/COX-2 selectivity of nimesulide based on pharmacokinetic and pharmacodynamic data. Their results suggest that a dose of 1.5 mg/kg bwt at a dosing interval of 12-24 hours may produce adequate clinical effects.

However, they found that at this particular dose, the concentration in the animal exceeded the *in vitro* IC₅₀, the COX-1/COX-2 selectivity is lost leading to side-effects due to COX-1 inhibition.

Nine adult horses receiving a single i.v dose of 0.5, 1.5 and 3 mg/kg bwt tramadol showed peak plasma concentrations of 454 ± 101.6 , 1086.7 ± 330.7 and 1697.9 ± 406.1 ng/ml, respectively. The CL, V_d and t_{1/2} also ranged from 24.6 to 25 ml/min/kg, 2.66 to 3.33 L/kg and 2.17 to 3.05 hours respectively depending on the dose administered. Furthermore, horses were found to metabolize tramadol to O-desmethyltramadol preferably than N-desmethyltramadol. O-desmethyltramadol was present at a concentration of 3.9 ± 1.9 , 9.6 ± 4.8 and 12.9 ± 5.2 ng/ml, respectively for the different doses administered (Knych *et al.*, 2012).

6.5. Metabolism and pharmacokinetics of celecoxib and etoricoxib *in vivo*

A HPLC-MS/MS-based technique is both rapid and easy and it has been found to be a highly suitable chemical tool to analyse for metabolites in biological samples such as urine, faeces and plasma and moreover to study the pharmacokinetics of COX-2 inhibitors (Lutz Brautigam, 2001). Similar to steroids, non steroidal anti inflammatory drugs are most widely studied in horses because of their importance as therapeutic agents. In the equine, aspirin (acetyl salicylic acid) is rapidly hydrolyzed to salicylic acid and it is the predominant urinary metabolite (Beaumier *et al.*, 1987). Phenylbutazone is the most common NSAID used in horses for musculoskeletal pain (Kallings *et al.*, 1999b). Phenylbutazone is a COX-1 and COX-2 inhibitor and it is metabolized via oxidation to form oxyphenylbutazone, though γ -hydroxy-phenylbutazone, the metabolite reported in plasma within 1-10 hours after administration in horses (Tobin *et al.*, 1986). Flunixin is another traditional NSAID

used more frequently in horses for colic and soft tissue inflammation. This NSAID has been found to form a hydroxy metabolite which is also active and has an analgesic activity for about 12.8 hours (Houdeshell & Hennessey, 1977). Caffeine, the most commonly used stimulant in humans undergoes demethylation in horses to form the dimethylated xanthine namely, theophylline, together with traces of theobromine and paraxanthine (Clarke & Moss, 1976). The metabolic profile of naproxen has been studied extensively in horses which forms O-desmethyl naproxen and it is detected in both plasma and urine (Young & Yeow, 1983).

The COX-2 inhibitors are relatively new group of anti-inflammatory agents which do not disturb the house keeping function of the COX-1 enzymes, thus, they are found to be tolerated well by gastrointestinal system. The pharmacokinetics of celecoxib and etoricoxib have been extensively studied previously (Yuan & Hunt, 2007). Celecoxib exhibits analgesic, antipyretic and anti-inflammatory properties by inhibiting prostaglandin synthesis and its analgesic effect is mainly due to its peripheral action at the site of pain and it is most effective when the pain is related to inflammation (Antoniou *et al.*, 2007). Due to its poor solubility, the oral bioavailability of celecoxib has been found to be low (Babu *et al.*, 2002a).

Paulson *et al.* (2001) have shown that celecoxib reached a maximum concentration within 1 hour after oral administration in dogs with a reported t_{\max} of 2.5 hours. In fasted humans, a higher t_{\max} of 3.5 hours was reported for celecoxib after a high fat meal. Following a 200 mg oral dose of celecoxib to human subjects, a t_{\max} of ~2.9 hours, C_{\max} of $\sim 806 \pm 411$ ng/ml, terminal half-life ($t_{1/2}$) of 7.6 – 15.2 hours were reported (Werner *et al.*, 2002; Abdel-Hamid *et al.*, 2001; Itthipanichpong *et al.*, 2005;

Davies *et al.*, 2000;). Similarly, C_{\max} of 0.67 ± 0.17 hours and a $t_{1/2}$ of 3.7 – 14 hours were reported for dogs and cats (Paulson *et al.*, 2000; Paulson *et al.*, 2001). Kvaternick *et al.* (2007) studied the pharmacokinetics of firocoxib following daily oral administration in horses and they have reported a C_{\max} of 75 ng/ml, t_{\max} of 3.9 hours and a terminal half-life of 30 hours. Steady state plasma concentration are attained within 5 days after starting therapy and the metabolites found in the circulation have no COX-2 activity (Pfizer Ltd, 2012). Celecoxib is bound to plasma albumin ($\approx 97\%$) and distributed in the tissues extensively (Kucab *et al.*, 2005).

Human study has predicted that celecoxib metabolism is primarily mediated via cytochrome P450C9 and three metabolites namely hydroxycelecoxib, carboxycelecoxib and its corresponding glucuronide have been identified in plasma (Gong *et al.*, 2012). Zhang *et al.* (2000) in their study of the metabolism of celecoxib, reported that the drug is extensively metabolized with less than 2% of the administered dose is eliminated unchanged in urine and faeces of rabbits. Furthermore, they have characterized three phase I and four phase II metabolites using LC/MS/MS in conjunction with radio labelled profiling. Paulson *et al.*, (2001) have reported that celecoxib is predominantly eliminated as metabolites in urine and faeces, with 57% of the dose excreted in faeces and 27% excreted in the urine in humans. Moreover, they showed that carboxycelecoxib was the main metabolite in both urine and faeces.

Etoricoxib produces dose-dependent inhibition of COX-2 without inhibition of COX-1 across the therapeutic dose range (Dallob *et al.*, 2003). Agrawal *et al.*, (2003a) reported that the peak plasma concentration of etoricoxib reached within 1 hour of oral administration. Etoricoxib has been found to be bound to plasma protein ($\approx 92\%$) and after a 120 mg daily dose, the area under the plasma time curve (AUC) was 37.8 μ g/ml

(Agrawal *et al.*, 2004). The drug has been found to be extensively metabolized by CYP 3A4 and to a lesser extent by CYP2D6 (Kassahun *et al.*, 2001). Five metabolites, namely 6'-hydroxymethyl etoricoxib, 6'-carboxy etoricoxib, etoricoxib-1-N-oxide, 6'-hydroxymethyl-etoricoxib-1'-N-oxide and 6'-hydroxymethyl etoricoxib glucuronide have been identified in urine and none of the metabolites have significant pharmacological activity (Chauret *et al.*, 2001).

A radiolabelled etoricoxib administration study by Rodrigues *et al.*, (2003) showed that an amount of 70% of the dose is excreted in urine and 20% in faeces as metabolites, and less than 2% excreted as unchanged drug. They also reported that 6'-carboxy etoricoxib was the major metabolite excreted in urine and faeces and 6'-hydroxymethyl etoricoxib, etoricoxib-1'-N-oxide, the 1'-N-oxide of 6'-hydroxymethyl etoricoxib and the glucuronide conjugate of the 6'-hydroxymethyl etoricoxib comprised $\leq 7\%$ when the drug was administered as a radioactive compound in 0 – 24 hours urine.

Likewise, Agrawal *et al.* (2001) reported that the plasma clearance of etoricoxib and its metabolites was ≈ 50 ml/min and a plasma half-life of about 22 hours, suggesting a once a daily dose. Furthermore, they concluded that less than 1% of the administered drug was recovered intact in urine, indicating that renal excretion has a minimal role in the excretion of etoricoxib. However, the metabolites of etoricoxib are mostly excreted in urine.

The pharmacokinetics of etoricoxib does not have a significant effect on the age and gender of the subjects employed in the study. In contrast, the pharmacokinetics of the etoricoxib is contradicted in patients with renal creatinine clearance < 30 ml/min (Merck Sharp & Dohme Limited, 2012). A three-part study of either intravenous, oral administration or a multiple oral dose of etoricoxib has showed pharmacokinetics and

absorption characteristics which were linear over the dose and route studied (Agrawal *et al.*, 2003b).

6.5. Metabolism of celecoxib and etoricoxib *in vitro*

Human liver-derived *in vitro* systems for prediction of drug clearance and metabolism was described ~ 40 year ago (Rane *et al.*, 1977). The synthesis of drug metabolites have become a tedious and cumbersome process and thus the use of *in vitro* technology has made it possible to produce metabolites with high degree of purity within a few hours. *In vivo* experiments also require ethical clearance for the use of animals for research work and they are often lengthy involving administration protocols and timescales. On the other hand *in vitro* experiments do not require live animals and the final extracts are cleaner and can be fit into tailor made time scales (Scarth *et al.*, 2010b). Thus, various *in vitro* systems such as liver microsomes, hepatocytes and precision cut liver slices are available to study drug clearance and metabolism (Obach *et al.*, 1997).

The *in vitro* drug metabolic study is wide spread in drug development industry and has not been fully utilized in sport drug testing laboratories (Yuan *et al.*, 2002). Very few publications are available about the use of horse liver and liver fractions in metabolism studies and they have been mostly on the study of the phase I metabolism of anabolic-androgenic steroids. Leung *et al.*, (2005) studied the *in vitro* biotransformation of clostebol acetate with horse liver microsomes and reported the formation of six metabolites while they found only three metabolites in urine. The *in vitro* assay produced cleaner byproducts of clostebol which helped in detection of minor metabolites compared to dirty urine matrix which hindered the detection of less concentration of minor metabolites. Clostebol acetate is used to enhance the

performance in race horses. Similarly Ho *et al.*, (2005) studied the metabolism of methenolone using horse liver microsomes and detected seven metabolites for this steroid. Again, Ho *et al.*, (2007) studied the metabolism of turinabol an oral anabolic steroid using horse liver microsomes. The metabolites were detected by GC-MS after trimethylsilylation. They reported that turinabol undergoes hydroxylation at sites C6, C16 and C20 of the molecule to form five metabolites. The structures of all the metabolites were tentatively identified by mass spectral interpretation.

Finally, Scarth *et al.*, (2010) studied the metabolism of stanazolol by LC-MS using equine liver/lung microsomes and S9 fractions and found a number of phase I metabolites previously unreported in the equine. The preparation of horse liver microsomes from fresh horse liver involved time consuming steps, which led Wong *et al.*, (2011) to use fresh horse liver slices to study the metabolism of five anabolic steroids – turinabol, metholone acetate, androst-4-ene-3,4,17-trione, testosterone and epitestosterone. They report that the assay was time saving as well as the whole liver tissue produced more number of metabolites than using liver microsomes reported by other others. Thus the *in vitro* studies are used in equine drug testing laboratories to compliment the *in vivo* studies (Ho *et al.*, 2007b).

A NADPH fortified human liver microsome incubation study previously reported the formation of one major metabolite of celecoxib which was identified as hydroxy celecoxib with no carboxy celecoxib formation (Tang *et al.*, 2000). Similarly Sandberg *et al.*, (2002) studied the metabolism of celecoxib using human liver microsomes in the presence of NADPH and report that celecoxib produced one metabolite namely OH-celecoxib and the formation of this metabolite was inhibited if the concentration of acetonitrile used to dissolve celecoxib was more than 10%. Carboxycelecoxib was not

present in the incubation assay. They further report that carboxy celecoxib was not formed with OH-celecoxib incubated as a substrate even at high concentrations, whereas, addition of liver cytosol to the incubation medium enhanced the oxidation of OH-celecoxib to form COOH-celecoxib. Thus their experiments concluded that COOH-celecoxib formation was clearly dependent on the presence of cytosol in the microsomal assay.

Chauret *et al.*, (2001) studied the *in vitro* metabolism of etoricoxib in human liver microsomes and they demonstrated the formation of two oxidative metabolites, namely 6'-hydroxymethyl etoricoxib as the major metabolite and etoricoxib-1'-N-oxide as minor metabolite and further, when incubated with suspended haepatocytes, etoricoxib formed the third metabolite namely 6'-carboxy etoricoxib. The same group of workers further studied the metabolism of etoricoxib and identified a fourth metabolite in canine haepatocytes having a mass of 192 daltons, higher than that of the parent etoricoxib and this metabolite was confirmed to be 6'-hydroxymethyl glucuronated metabolite. The incubation of the synthetic 6'-hydroxymethyl analogue with dog haepatocytes formed 6'-hydroxymethyl glucuronated metabolite, which allowed its characterization. Kassahun *et al.*, (2001) found that CYP3A4 plays a major role, approximately 40 to 90% of the total 6'-methyl hydroxylase activity in the metabolism of etoricoxib; however, other CYPs like the CYP2D6, CYP2C9 also have a role to play as well. Moreover ketoconazole, sulfaphenazole, quinidine and troleandomycin, selective inhibitors of CYP3A were shown to decrease the rate of metabolite production with human liver microsome incubation. They further report that etoricoxib with NADPH-fortified human liver microsomes formed one major peak which was identified by LC/MS as 6'-methyl hydroxyl metabolite, traces of etoricoxib-1-N-oxide as minor

metabolite and the complete absence of 6'-carboxy metabolite which could be detected in human urine and faeces.

6.7. Concluding remarks

The results presented in this study have clearly established that celecoxib and etoricoxib are safe drugs on the gastrointestinal system of the horse with virtually no adverse side effects on the clinical biochemical and haematological parameters following oral administration at physiological dose. Both drugs are predominantly metabolized in the liver. Celecoxib has been found to be not fully absorbed by the gastrointestinal tract, thus it was eliminated as a major component in the faeces of the horses. In contrast, etoricoxib undergoes extensive metabolism and only low levels of etoricoxib are excreted in urine and faeces. Celecoxib or its metabolites could be detected for up to 96 hours and etoricoxib or its metabolites were detected for a maximum of 120 hours following oral administration. In vitro studies employing either horse hepatocytes or camel liver slices have also demonstrated that both coxibs can be metabolized producing almost similar metabolites as seen in vivo studies. Thus, this study draws a safety line for the Veterinary Practitioners, trainers, owners and the horse racing industry of the safe usage of the drug as a genuine therapeutic medication for the treatment of pain and the withdrawal time period for a drug free sport.

CHAPTER -7

CONCLUSIONS AND SCOPE FOR FUTURE STUDIES

7.1 Conclusions

Non-steroidal anti-inflammatory drugs or NSAIDs play a major role in reducing pain and inflammation both in humans and animals. There are different types of NSAIDs, some of which induce several side effects including bleeding and ulceration. In the pharmaceutical industry research is still going on in producing safer NSAIDs. Two recently developed NSAIDs are celecoxib and etoricoxib and they have been shown to exert safe analgesic effects in humans. However, no such work was done in large animals such as horse which usually experience joint and muscle pain, especially during racing.

This study investigated their side effects, metabolism and elimination of these two coxib analgesics in six retired race horses. The results show that oral administration of both COX-2 inhibitors at physiological doses (2 mg/kg bwt for celecoxib or 0.5 mg/kg, 1 mg/kg and 2 mg/kg bwt of etoricoxib had no adverse side effects on either the stomach mucosa or on blood borne parameters including RBC, WBC and platelet counts as well as on liver and kidney function enzymes and plasma anions and cations, except for a small transient increase in creatinine with etoricoxib. Following oral administration, either drug is metabolized in the animals in a time dependent manner producing several metabolites as well as the parent drug in urine, faeces and plasma. The liver seems to be the main route in metabolizing each drug. Both the parent drug and metabolites are completely eliminated from the animals within five days following oral administration.

In vitro studies involving commercially purchased horse liver microsomes and fresh camel liver fragments from the local abattoir were also used to investigate the

metabolism of either celecoxib or etoricoxib. The results show that horse liver microsomes or camel liver fragments can metabolize each drug over a period of 30 to

180 minutes following incubation to form either none or one or two metabolites, compared to the whole animal.

In conclusion, the results have indicated that the horse can safely tolerate both coxibs, to treat pain and without any major side effects. Moreover, the pharmacokinetic data suggest that the animal can harbor both the parent drugs and metabolites for up to 5 days following oral administration. This latter finding is of paramount importance for the horse racing fraternity and the industry especially if animals are routinely given coxib analgesic prior to a race.

7.2. Scope for future studies

1. It is possible to investigate higher pharmacological doses of each drug in the animals and to repeat the same experiments as described in the study, similar to those used in rats and dogs. These high doses can be administered orally to the animals over time. Samples of blood, urine and faeces will be collected for analysis of certain blood borne biochemical parameters, the parent drug and other metabolites using conventional techniques employed in this study. In addition, it is possible to undertake endoscopic studies to see if the high dose of each drug can induce ulcer of the stomach.
2. It is also proposed to investigate other related coxibs both at therapeutic and pharmacological doses in horses and camels, two large animals which are normally used in racing. There are other related coxibs which have not yet been investigated in large animals such as horses and to determine the analgesic effects. Similar to this study, it is possible to employ one or possibly two different coxibs for comparison. Animals will be given each drug orally

employing different concentrations. Samples of blood, urine and faeces will be collected for analysis using similar methods employed in this study and measure similar blood borne parameters, the parent drug and their metabolites.

3. In the present study, each drug was eliminated in faeces without being metabolized suggesting that they are not properly absorbed by the gut. It may be possible to do uptake experiments using intestinal vesicles. The study can employ small animals such as rats, mice and rabbit and such large animals as the camel, sheep as well as horse whenever the tissues are available. This will involve the use of radioactive coxibs. Typically, each radioactive coxib can be incubated with brush border vesicle of the small and large intestine of rats, mice or a guinea pig over time. The uptake of each drug can be determined using scintillation analysis and plotted as time dependant curves. In addition, it may be possible to measure both the parent drug and metabolites during these experiments. These results may provide important information on the role of brush border vesicles in both absorption and metabolism of each coxibs.
4. Ideally, it is important to measure the effect and metabolism of the two coxibs after a race. Blood is first collect before the race. The animals are given the drug prior to the race and blood, faecal and urine samples collected at different time intervals after the race. These experiments may indicate whether vigorous exercise can help to metabolize and eliminate the drug faster compared to non exercised animals. In these proposed series of experiments, the urine, faeces and

blood samples will be analyzed for blood borne biochemical parameters, the parent drug as well its metabolites using conventional and chemical techniques employed in this study.

5. There is some evidence that the two coxibs can reduce blood triglycerides and cholesterol, at least in humans. It may be possible to repeat some of the experiments but concentrate more on the lipids. Lipids including HDL, LDL, CRP, cholesterol and total triglycerides may indicate signs of fatigue, distress and if possible cardiac events. These different lipids can be measured in blood using conventional biochemical analyses employing the auto-analyser. Again, different doses of coxib may induce different effect on each lipid. It may be possible to use low as well as high dose of each coxib during these proposed studies.

CHAPTER – 8

REFERENCES

REFERENCES

Reference List

Abdel-Hamid, M., Novotny, L., & Hamza, H. (2001). Liquid chromatographic-mass spectrometric determination of celecoxib in plasma using single-ion monitoring and its use in clinical pharmacokinetics. *Journal of Chromatography B* **753**, 401-408.

Abramovitz, M. & Metters, K. M. (1998). Chapter 22. Prostanoid Receptors. In *Annual Reports in Medicinal Chemistry*, ed. James, A. B., pp. 223-232. Academic Press.

Abu-Kishk, I., Goldman, M., Mordish, Y., Berkovitch, M., & Kozer, E. (2010). Transient renal insufficiency following dipyrene overdose. *Archives of Disease in Childhood* **95**, 233-234.

Adams, H. R. (2000). *Veterinary pharmacology and therapeutics*, 8th edition ed., pp. 433-452. Blackwell Publishing Professional.

Agrawal, N. G., Matthews, C. Z., Rose, M. J., Woolf, E. J., Porras, A. G., Mazina, K. E., Farmer, J., Musser, B. J., Dynder, A. L., Lasseter, K. C., Hunt, T. L., Mccrea, J. B., Schwartz, J. I., & Gottesdiener, K. G. (2002). Single- and multiple-dose pharmacokinetics of etoricoxib, a highly selective inhibitor of cyclooxygenase-2, in healthy subjects. *Clinical Pharmacology & Therapeutics* **71**, 50.

Agrawal, N. G. B., Matthews, C. Z., Mazenko, R. S., Kline, W. E., Woolf, E. J., Porras, A. G., Geer, L. A., Wong, P. H., Cho, M. H., Cote, J., Marbury, T. C., Moncrief, J. W., Alcorn, H., Swan, S., Sack, M. R., Robson, R. A., Petty, K. J., Schwartz, J. I., & Gottesdiener, K. M. (2004). Pharmacokinetics of etoricoxib in patients with renal impairment. *Journal of Clinical Pharmacology* **44**, 48-58.

Agrawal, N. G. B., Porras, A. G., Matthews, C. Z., Rose, M. J., Woolf, E. J., Musser, B. J., Dynder, A. L., Mazina, K. E., Lasseter, K. C., Hunt, T. L., Schwartz, J. I., Mccrea, J. B., & Gottesdiener, K. M. (2003a). Single- and multiple-dose pharmacokinetics of etoricoxib, a selective inhibitor of cyclooxygenase-2, in man. *Journal of Clinical Pharmacology* **43**, 268-276.

Agrawal, N. G. B., Porras, A. G., Matthews, C. Z., Woolf, E. J., Miller, J. L., Mukhopadhyay, S., Neu, D. C., & Gottesdiener, K. M. (2001). Dose proportionality of oral etoricoxib, a highly selective cyclooxygenase-2 inhibitor, in healthy volunteers. *Journal of Clinical Pharmacology* **41**, 1106-1110.

Agrawal, N. G. B., Rose, M. J., Matthews, C. Z., Woolf, E. J., Porras, A. G., Geer, L. A., Larson, P. J., Cote, J., Dilzer, S. C., Lassetter, K. C., Alam, I., Petty, K. J., & Gottesdiener, K. M. (2003b). Pharmacokinetics of etoricoxib in patients with hepatic impairment. *Journal of Clinical Pharmacology* **43**, 1136-1148.

Al Sukhun, J., Al Sukhun, S., Penttila, H., Ashammakhi, N., & Al Sukhun, R. (2012). Preemptive Analgesic Effect of Low Doses of Celecoxib Is Superior to Low Doses of Traditional Nonsteroidal Anti-Inflammatory Drugs. *Journal of Craniofacial Surgery* **23**, 526-529.

Alavijeh, M. S., Chishty, M., Qaiser, M. Z., & Palmer, A. M. (2005). Drug metabolism and pharmacokinetics, the blood-brain barrier, and central nervous system drug discovery. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* **2**.

Alexander Valverde, D. D. D. a. C. I. G. Dr. m. v. M. (2005). Painmanagement in horses and farmanimals. *Journal of Veterinary Emergency and Critical Care* **15**, 295-307.

Altinkaynak, K., Suleyman, H., & Akcay, F. (2003). Effect of nimesulide, rofecoxib and celecoxib on gastric tissue glutathione level in rats with indomethacin-induced gastric ulcerations. *Polish Journal of Pharmacology* **55**, 645-648.

Andersohn, F., Suissa, S., & Garbe, E. (2006). Use of first- and second-generation cyclooxygenase-2-selective nonsteroidal antiinflammatory drugs and risk of acute myocardial infarction. *Circulation* **113**, 1950-1957.

Anon (2000). Why bother about cytochrome P450 enzymes? *Drug and Therapeutics Bulletin* **38**, 93-96.

Anonymous. Etoricoxib Proves to be Effective Worldwide. 2012.
Ref Type: Internet Communication

Anonymous. Arcoxia 30mg, 60 mg, 90 mg & 120 mg Film-coated Tablets. Available from URL:<http://www.emc.medicines.org.uk> . 2013.
Ref Type: Electronic Citation

Ansary, M. (1983). Phenylbutazone in the Therapeutics of Horses. *Annales de Medecine Veterinaire* **127**, 383-388.

Antman, E. M., Bennett, J. S., Daugherty, A., Furberg, C., Roberts, H., & Taubert, K. A. (2007). Use of nonsteroidal Antiinflammatory drugs an update for clinicians - A scientific statement from the American Heart Association. *Circulation* **115**, 1634-1642.

Antoniou, K., Malamas, M., & Drosos, A. A. (2007). Clinical pharmacology of celecoxib, a COX-2 selective inhibitor. *Expert Opinion on Pharmacotherapy* **8**, 1719-1732.

Anzenbacher, P. (2001). Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences* **58**, 737-747.

Armstrong, S. & Lees, P. (1999). Effects of R and S enantiomers and a racemic mixture of carprofen on the production and release of proteoglycan and prostaglandin E-2 from equine chondrocytes and cartilage explants. *American Journal of Veterinary Research* **60**, 98-104.

Armstrong, S., Tricklebank, P., Lake, A., Frean, S., & Lees, P. (1999). Pharmacokinetics of carprofen enantiomers in equine plasma and synovial fluid - a comparison with ketoprofen. *Journal of Veterinary Pharmacology and Therapeutics* **22**, 196-201.

Aspinall, V. (2009). *Essentials of Veterinary Anatomy and Physiology* Elsevier Ltd.London.

Ault, A. (1998). First COX-2 inhibitor clears initial FDA hurdle. *Lancet* **352**, 1912.

Babu, Gouri Shankar, & Hima Sankar (2002a). PrFont34Bin0BinSub0Frac0Def1Margin0Margin0Jc1Indent1440Lim0Lim1Development Of Dissolution Medium For A Poorly Water Soluble Drug, Celecoxib. *Indian Journal of Pharmaceutical Sciences* **64**, 588-591.

Babu, Shankar, & Sankar (2002b). Development of dissolution medium for a poorly water soluble drug, Celecoxib. *Indian Journal of Pharmaceutical Sciences* **64**, 588-591.

Back, W., MacAllister, C., van Heel, M., Pollmeier, M., & Hanson, P. (2009). The use of force plate measurements to titrate the dosage of a new COX-2 inhibitor in lame horses. *Equine Veterinary Journal* **41**, 309-312.

Baggot, J. D. (1995). Practical Implications of Pharmacokinetic and Pharmacodynamic (Pk/Pd) Modeling of Drug Dose Regimens. *Equine Veterinary Journal* **27**, 245-246.

Baracho, N. C. D., Guizelli, G. P., Carmello, B. L., Sanches, D. D., Silva, F. M. C., dos Reis, J. M., & de Britoi, J. (2009). Cardiovascular and hematologic effects produced by chronic treatment with etoricoxib in normotensive rats. *Acta Cirurgica Brasileira* **24**, 206-210.

Baraf, H. S. B., Fuentealba, C., Greenwald, M., Brzezicki, J., O'Brien, K., Soffer, B., Bird, S., Reicin, A. S., & Curtis, S. P. (2004). Tolerability and effectiveness of etoricoxib compared to diclofenac sodium in patients with osteoarthritis: A randomized controlled study (EDGE trial). *Arthritis and Rheumatism* **50**, S346-S347.

Baraf, H. S. B., Fuentealba, C., Greenwald, M., Brzezicki, J., O'Brien, K., Soffer, B., Polis, A., Bird, S., Kaur, A., Curtis, S. P., & Edge, S. G. (2007). Gastrointestinal side effects of etoricoxib in patients with osteoarthritis: Results of the etoricoxib versus diclofenac sodium gastrointestinal tolerability and effectiveness (EDGE) trial. *Journal of Rheumatology* **34**, 408-420.

Baylis, C. & Brenner, B. M. (1978). Mechanism of Glucocorticoid-Induced Increase in Glomerular-Filtration Rate. *American Journal of Physiology* **234**, F166-F170.

Beaumier, P. M., Fenwick, J. D., Stevenson, A. J., Weber, M. P., & Young, L. M. (1987). Presence of Salicylic-Acid in Standard-Bred Horse Urine and Plasma After Various Feed and Drug Administrations. *Equine Veterinary Journal* **19**, 207-213.

Behal, N., Kanwar, S. S., Sharma, P., & Sanyal, S. (2009). Effect of non-steroidal anti-inflammatory drug etoricoxib on the hematological parameters and enzymes of colon and kidney. *Nutricion Hospitalaria* **24**, 326-332.

Bennett, W. M., Henrich, W. L., & Stoff, J. S. (1996). The renal effects of nonsteroidal anti-inflammatory drugs: Summary and recommendations. *American Journal of Kidney Diseases* **28**, S56-S62.

Bensen, W. G., Zhao, S. Z., Burke, T. A., Zabinski, R. A., Makuch, R. W., Maurath, C. J., Agrawal, N. M., & Geis, G. S. (2000). Upper gastrointestinal tolerability of celecoxib, a COX-2 specific inhibitor, compared to naproxen and placebo. *Journal of Rheumatology* **27**, 1876-1883.

Berg, K. J., Gjellestad, A., Nordby, G., Rootwelt, K., Djoseland, O., Fauchald, P., Mehl, A., Narverud, J., & Talseth, T. (1989). Renal Effects of Trimethoprim in Cyclosporin-Treated and Azathioprine-Treated Kidney-Allografted Patients. *Nephron* **53**, 218-222.

Bergstroem, S., Danielsson, H., & Samuelsson, B. (1964). The Enzymatic Formation of Prostaglandin E₂ from Arachidonic Acid Prostaglandins and Related Factors 32. *Biochim Biophys Acta* **90**.

Bernd Driessen (2007). Pain: From Diagnosis to Effective Treatment. *Clinical Techniques in Equine Practice*.

Berschneider, F. & Richter, W. (1980). History and Definition of Doping in Horse Racing. *Monatshefte fur Veterinarmedizin* **35**, 739-742.

Bienhoff, S. E., Smith, E. S., Roycroft, L. M., & Roberts, E. S. (2012). Efficacy and Safety of Deracoxib for Control of Postoperative Pain and Inflammation Associated with Soft Tissue Surgery in Dogs. *Veterinary Surgery* **41**, 336-344.

Bingham, C. O., Sebba, A. I., Rubin, B. R., Ruoff, G. E., Kremer, J., Bird, S., Smugar, S. S., Fitzgerald, B. J., O'Brien, K., & Tershakovec, A. M. (2007). Efficacy and safety of etoricoxib 30 mg and celecoxib 200 mg in the treatment of osteoarthritis in two identically designed, randomized, placebo-controlled, non-inferiority studies. *Rheumatology* **46**, 496-507.

Birbara, C. A., Puopolo, A. D., Munoz, D. R., Sheldon, E. A., Mangione, A., Bohidar, N. R., & Geba, G. P. (2003). Treatment of chronic low back pain with etoricoxib, a new cyclo-oxygenase-2 selective inhibitor: improvement in pain and disability--a randomized, placebo-controlled, 3-month trial. *J.Pain* **4**, 307-315.

Blackshear, J. L., Napier, J. S., Davidman, M., & Stillman, M. T. (1985). Renal complications of nonsteroidal antiinflammatory drugs: Identification and monitoring of those at risk. *Seminars in Arthritis and Rheumatism* **14**, 163-175.

Bombardier, C., Laine, L., Reicin, A., Shapiro, D., Burgos-Vargas, R., Davis, B., Day, R., Ferraz, M. B., Hawkey, C. J., Hochberg, M. C., Kvein, T. K., & Schnitzer, T. J. (2000). Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* **343**, 1520-1528.

Bonica JJ (1979). Editorial The need of a taxonomy. *Pain* **6**, 247-252.

Botting, R. & Ayoub, S. S. (2005). COX-3 and the mechanism of action of paracetamol/acetaminophen. *Prostaglandins Leukotrienes and Essential Fatty Acids* **72**, 85-87.

Bozina, Bradamante, & Lovric (2009). Genetic Polymorphism of metabolic enzymes P450 (CYP) as a susceptibility factor for drug response, toxicity, and cancer risk. *Archives of Industrial Hygiene and Toxicology* **60**, 217-242.

Braeutigam, L., Nefflen, J. U., & Geisslinger, G. (2003). Determination of etoricoxib in human plasma by liquid chromatography-tandem mass spectrometry with electrospray ionisation. *Journal of Chromatography B* **788**, 309-315.

Brautigam, L., Nefflen, J. U., & Geisslinger, G. (2003). Determination of etoricoxib in human plasma by liquid chromatography-tandem mass spectrometry with electrospray ionisation. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **788**, 309-315.

Brautigam, L., Vetter, G., Tegeder, I., Heinkele, G., & Geisslinger, G. (2001). Determination of celecoxib in human plasma and rat microdialysis samples by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B* **761**, 203-212.

Bresalier, R. S., Sandler, R. S., Quan, H., Bolognese, J. A., Oxenius, B., Horgan, K., Lines, C., Riddell, R., Morton, D., Lanus, A., Konstam, M. A., & Baron, J. A. (2005). Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* **352**, 1092-1102.

Brink, P., DeGraves, F., Ravis, W. R., Johansen, D., Campbell, J. D., & Duran, S. H. (1998). Stereospecific pharmacokinetics of free and protein-bound ketoprofen in serum and synovial fluid of horses after intravenous and intramuscular administration. *American Journal of Veterinary Research* **59**, 739-743.

Brum Junior, L., Catia Ceni, D., Fronza, M., de Oliveira, P. R., & Dalmora, S. L. (2006). Validation of an LC-tandem MS/MS method for the determination of etoricoxib in human plasma and pharmaceutical formulations. *Journal of Liquid Chromatography & Related Technologies* **29**, 123-135.

Brune, K. & Hinz, B. (2004). Selective cyclooxygenase-2 inhibitors: similarities and differences. *Scandinavian Journal of Rheumatology* **33**, 1-6.

Brunson, D. B. & Majors, L. J. (1987). Comparative Analgesia of Xylazine, Xylazine Morphine, Xylazine Butorphanol, and Xylazine Nalbuphine in the Horse, Using Dental Dolorimetry. *American Journal of Veterinary Research* **48**, 1087-1091.

Burns, J. J. & Conney, A. H. (1964). Therapeutic Implications of Drug Metabolism. *Seminars in hematology* **1**, 375-400.

Busby, W. F., Ackermann, J. M., & Crespi, C. L. (1999). Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on in vitro activities of cDNA-expressed human cytochromes P-450. *Drug Metabolism and Disposition* **27**, 246-249.

Cambridge, A. J., Tobias, K. M., Newberry, R. C., & Sarkar, D. K. (2000). Subjective and objective measurements of postoperative pain in cats. *Journal of the American Veterinary Medical Association* **217**, 685-690.

Cambridge, H., Lees, P., Hooke, R. E., & Russell, C. S. (1991). Antithrombotic Actions of Aspirin in the Horse. *Equine Veterinary Journal* **23**, 123-127.

Cannon, C. P., Curtis, S. P., Bolognese, J. A., & Laine, L. (2006). Clinical trial design and patient demographics of the multinational etoricoxib and diclofenac arthritis long-term (MEDAL) study program: Cardiovascular outcomes with etoricoxib versus diclofenac in patients with osteoarthritis and rheumatoid arthritis. *American Heart Journal* **152**, 237-245.

Capone, M. L., Tacconelli, S., Di Francesco, L., Sacchetti, A., Sciulli, M. G., & Patrignani, P. (2007). Pharmacodynamic of cyclooxygenase inhibitors in humans. *Prostaglandins & Other Lipid Mediators* **82**, 85-94.

Chan, F. K. L., Lan, A., Scheiman, J., Berger, M. F., Nguyen, H., & Goldstein, J. L. (2010). Celecoxib versus omeprazole and diclofenac in patients with osteoarthritis and rheumatoid arthritis (CONDOR): a randomised trial. *Lancet* **376**, 173-179.

Chandrasekharan, N. V., Dai, H., Roos, K. L. T., Evanson, N. K., Tomsik, J., Elton, T. S., & Simmons, D. L. (2002). COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 13926-13931.

Chauhan, B., Shimpi, S., & Paradkar, A. (2005). Preparation and characterization of etoricoxib solid dispersions using lipid carriers by spray drying technique. *AAPS PharmSciTech*. **6**, E405-E412.

Chauret, N., Gauthier, A., Martin, J., & Nicoll-Griffith, D. A. (1997). In vitro comparison of cytochrome P450-mediated metabolic activities in human, dog, cat, and horse. *Drug Metabolism and Disposition* **25**, 1130-1136.

Chauret, N., Gauthier, A., & Nicoll-Griffith, D. A. (1998). Effect of common organic solvents on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes. *Drug Metabolism and Disposition* **26**, 1-4.

Chauret, N., Yergey, J. A., Brideau, C., Friesen, R. W., Mancini, J., Riendeau, D., Silva, J., Styhler, A., Trimble, L. A., & Nicoll-Griffith, D. A. (2001). In vitro metabolism considerations, including activity testing of metabolites, in the discovery and selection of the COX-2 inhibitor etoricoxib (MK-0663). *Bioorganic & Medicinal Chemistry Letters* **11**, 1059-1062.

Chay, S., Nugent, T., Blake, J. W., Tobin, T., & Woods, W. E. (1982). The Pharmacology of Nonsteroidal Anti Inflammatory Drugs in the Horse Flunixin Meglumine Banamine. *Equine Practice* **4**, 16-23.

Chow, L. W. C., Loo, W. T. Y., & Toi, M. (2005). Current directions for COX-2 inhibition in breast cancer. *Biomedicine & Pharmacotherapy* **59**, S281-S284.

Chu, I. & Nomeir, A. A. (2006). Utility of mass spectrometry for in-vitro ADME assays. *Current Drug Metabolism* **7**, 467-477.

Claria, J. (2003). Cyclooxygenase-2 biology. *Current Pharmaceutical Design* **9**, 2177-2190.

Clarke, E. G. & Moss, M. S. (1976). A brief history of dope detection in racehorses. *Br J Sports Med* **10**, 100-102.

Clarke, R., Derry, S., & Moore, R. A. (2012). Single dose oral etoricoxib for acute postoperative pain in adults. *Cochrane database of systematic reviews (Online)* **4**.

Coffman, J. R. & Garner, H. E. (1972). Acute Laminitis. *Journal of the American Veterinary Medical Association* **161**, 1280-&.

Compston, J. E., Horton, L. W. L., & Laker, M. F. (1979). Vitamin-D Analogs and Renal-Function. *Lancet* **1**, 386.

Conlon, P. D. (1988). Nonsteroidal Drugs Used in the Treatment of Inflammation. *Veterinary Clinics of North America-Small Animal Practice* **18**, 1115-1131.

Cook, V. L., Meyer, C. T., Campbell, N. B., & Blikslager, A. T. (2009). Effect of firocoxib or flunixin meglumine on recovery of ischemic-injured equine jejunum. *American Journal of Veterinary Research* **70**, 992-1000.

Corveleyn, S., Deprez, P., VanderWeken, G., Baeyens, W., & Remon, J. P. (1996). Bioavailability of ketoprofen in horses after rectal administration. *Journal of Veterinary Pharmacology and Therapeutics* **19**, 359-363.

Crofford, L. J. (2000). Clinical Experience with Specific COX-2 Inhibitors in Arthritis. *Current Pharmaceutical Design* **6**, 1725.

Cryer, B., Li, C., Simon, L., Singh, G., Stillman, M., & Berger, M. (2011). GI-REASONS: A Novel 6-Month, Prospective, Randomized, Open-Label, Blinded End Point (PROBE) Trial. *American Journal of Gastroenterology* **106**, S85.

Curtis, S. P., Ng, J., Yu, Q., Shingo, S., Bergman, G., McCormick, C. L., & Reicin, A. S. (2004). Renal effects of etoricoxib and comparator nonsteroidal anti-inflammatory drugs in controlled clinical trials. *Clin. Ther.* **26**, 70-83.

DAGati, V. (1996). Does aspirin cause acute or chronic renal failure in experimental animals and in humans? *American Journal of Kidney Diseases* **28**, S24-S29.

Dallob, A., Hawkey, C. J., Greenberg, H., Wight, N., De Schepper, P., Waldman, S., Wong, P., DeTora, L., Gertz, B., Agrawal, N., Wagner, J., & Gottesdiener, K. (2003). Characterization of etoricoxib, a novel, selective COX-2 inhibitor. *Journal of Clinical Pharmacology* **43**, 573-585.

Dalmora, S. L., Sangoi, M. D. S., da Silva, L. M., Macedo, R. O., & Barth, T. (2008). Validation of a capillary zone electrophoresis method for the comparative determination of etoricoxib in pharmaceutical formulations. *Journal of Separation Science* **31**, 169-176.

Dannhardt (2002). COX-2 inhibition and the control of pain. *Curr Opin Investig Drugs* **3**, 1348-1358.

Dannhardt, G. & Kiefer, W. (2001). Cyclooxygenase inhibitors - current status and future prospects. *European Journal of Medicinal Chemistry* **36**, 109-126.

Dass, C. (2007). *Fundamentals of contemporary mass spectrometry* Wiley-Interscience.

Deeks, J. (2002). Efficacy, tolerability, and upper gastrointestinal safety of celecoxib for treatment of osteoarthritis and rheumatoid arthritis: systematic review of randomised controlled trials. *BRITISH MEDICAL JOURNAL* **325**, 619-623.

Delbeke, F. T., Landuyt, J., & Debackere, M. (1994). Disposition of Human Drug Preparations in the Horse .3. Orally-Administered Alclofenac. *Journal of Veterinary Pharmacology and Therapeutics* **17**, 353-358.

DiMaio Knych, H. K. & Stanley, S. D. (2008). Complementary DNA cloning, functional expression and characterization of a novel cytochrome P450, CYP2D50, from equine liver. *Biochemical Pharmacology* **76**.

Dixit, M. P., Doan, T., Kirschner, R. C., & Dixit, N. M. (2010). Significant Acute Renal Failure Due to Non-Steroidal Anti-Inflammatory Drugs: Inpatient Setting. *Pediatric Nephrology* **25**, 1836.

Dogterom, P. (1993). Development of A Simple Incubation System for Metabolism Studies with Precision-Cut Liver Slices. *Drug Metabolism and Disposition* **21**, 699-704.

Doucet, M. Y., Bertone, A. L., Hendrickson, D., Hughes, F., MacAllister, C., McClure, S., Reinemeyer, C., Rossier, Y., Sifferman, R., Vrins, A. A., White, G., Kunkle, B., Alva, R., Romano, D., & Hanson, P. D. (2008). Comparison of efficacy and safety of paste formulations of firocoxib and phenylbutazone in horses with naturally occurring osteoarthritis. *Javma-Journal of the American Veterinary Medical Association* **232**, 91-97.

Dowie, J. (1977). Turf - Social and Economic-History of Horse Racing - Vamplew, W. *Economic History Review* **30**, 356-357.

Driessen, B., Bauquier, S. H., & Zarucco, L. (2010). Neuropathic Pain Management in Chronic Laminitis. *Veterinary Clinics of North America-Equine Practice* **26**, 315-+.

Dubb, J. W., Stote, R. M., Familiar, R. G., Lee, K., & Alexander, F. (1978). Effect of Cimetidine on Renal-Function in Normal Man. *Clinical Pharmacology & Therapeutics* **24**, 76-82.

Dumasia, M. C. & Bruce, S. a. S. E. Detection of COX-2 selective NSAIDs: Biotransformation and Identification of in vivo metabolites of meloxicam in equine urine. D.W.Hill and W.T.Hill. Proceedings of the 14th International Conference of Racing Analysts and Veterinarians, 343-348. 2002.
Ref Type: Conference Proceeding

Dunn, M. J. (1984). *Nonsteroidal Anti Inflammatory Drugs and Renal Function*, pp. 411-428.

E.J.Love (2009). Assessment and management of pain in horses. *Equine Veterinary Education* **21**, 46-48.

Eddershaw, P. J. & Dickins, M. (1999). Advances in in vitro drug metabolism screening. *Pharmaceutical Science & Technology Today* **2**, 13-19.

Emami, J., Fallah, R., & Ajami, A. (2008). A rapid and sensitive HPLC method for the analysis of celecoxib in human plasma: application to pharmacokinetic studies. *Daru- Journal of Faculty of Pharmacy* **16**, 211-217.

Emery, P., Koncz, T., Pan, S., & Lowry, S. (2008). Analgesic effectiveness of celecoxib and diclofenac in patients with osteoarthritis of the hip requiring joint replacement surgery: a 12-week, multicenter, randomized, double-blind, parallel-group, double-dummy, noninferiority study. *Clinical Therapeutics* **30**, 70-83.

F.T.Delbeke *, J. L. M. D. (1995). Disposition of human drug preparations in the horse IV. Orally administered fenoprofen. *Journal of Pharmaceutical and Biomedical Analysis* **13**, 1041-1047.

Farkouh, M. E., Kirshner, H., Harrington, R. A., Ruland, S., Verheugt, F. W., Schnitzer, T. J., Burmester, G. R., Mysler, E., Hochberg, M. C., & Doherty, M. (2004). Comparison of lumiracoxib with naproxen and ibuprofen in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET), cardiovascular outcomes: randomized controlled trial. *Lancet* **364**, 675-684.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989). Electrospray Ionization for Mass-Spectrometry of Large Biomolecules. *Science* **246**, 64-71.

Ferreira, S. H., Moncada, S., & Vane, J. R. (1973). Prostaglandins and Mechanism of Analgesia Produced by Aspirin-Like Drugs. *British Journal of Pharmacology* **49**, 86-97.

Fink-Gremmels, J. (2008). Implications of hepatic cytochrome P450-related biotransformation processes in veterinary sciences. *European Journal of Pharmacology* **585**, 502-509.

Fiorucci, S., Distrutti, E., Mencarelli, A., Morelli, A., Lafor, S. A., Cirino, G., & Wallace, J. L. (2003). Evidence that 5-lipoxygenase and acetylated cyclooxygenase 2-derived eicosanoids regulate leukocyte-endothelial adherence in response to aspirin. *Br.J.Pharmacol.* **139**, 1351-1359.

FitzGerald, G. A. (2004). Coxibs and cardiovascular disease. *N Engl J Med* **351**, 1709-1711.

FitzGerald, G. A. & Patrono, C. (2001). The coxibs, selective inhibitors of cyclooxygenase-2. *N.Engl.J.Med.* **345**, 433-442.

Flecknell, P. (2008). Analgesia from a veterinary perspective. *British Journal of Anaesthesia* **101**, 121-124.

Friesen, R. W., Dub , D., Fortin, R., Frenette, R., Prescott, S., Cromlish, W., Greig, G. M., Kargman, S., Wong, E., Chan, C. C., Gordon, R., Xu, L. J., & Riendeau, D. (1996). Novel 1,2-diarylcyclobutenes: Selective and orally active cox-2 inhibitors. *Bioorganic & Medicinal Chemistry Letters* **6**, 2677-2682.

Fu, J. Y., Masferrer, J. L., Seibert, K., Raz, A., & Needleman, P. (1990). The Induction and Suppression of Prostaglandin-H2 Synthase (Cyclooxygenase) in Human Monocytes. *Journal of Biological Chemistry* **265**, 16737-16740.

Gans, K. R., Galbraith, W., Roman, R. J., Haber, S. B., Kerr, J. S., Schmidt, W. K., Smith, C., Hewes, W. E., & Ackerman, N. R. (1990). Antiinflammatory and Safety Profile of Dup-697, A Novel Orally Effective Prostaglandin Synthesis Inhibitor. *Journal of Pharmacology and Experimental Therapeutics* **254**, 180-187.

Gardner, D. S. (2006). Historical progression of racing performance in the Thoroughbred horse and man. *Equine Veterinary Journal* **38**, 581-583.

Garella, S. & Matarese, R. A. (1984). Renal Effects of Prostaglandins and Clinical Adverse-Effects of Nonsteroidal Anti-Inflammatory Agents. *Medicine* **63**, 165-181.

Gauthier, J. Y., Leblanc, Y., Cameron Black, W., Chan, C. C., Cromlish, W. A., Gordon, R., Kennedy, B. P., Lau, C. K., L ger, S., Wang, Z., Ethier, D., Guay, J., Mancini, J., Riendeau, D., Tagari, P., Vickers, P., Wong, E., Xu, L., & Prasit, P. (1996). Synthesis and biological evaluation of 2,3-diarylthiophenes as selective cox-2 inhibitors. part II: Replacing the heterocycle. *Bioorganic & Medicinal Chemistry Letters* **6**, 87-92.

Gerring, E. L., Lees, P., & Taylor, J. B. (1981). Pharmacokinetics of phenylbutazone and its metabolites in the horse. *Equine Veterinary Journal* **13**, 152-157.

Gn lner, H. g., Gill, J., bartter, F. C., & Dnsing, R. (1980). The role of the prostaglandin system in the regulation of renal function in normal women. *The American Journal of Medicine* **69**, 718-724.

Goldstein, J. L., Agrawal, N., Eisen, G. M., Stenson, W., Bello, A. E., Fort, J. G., & Boots, S. P. (2001). Significantly improved upper gastrointestinal (UGI) tolerability with celecoxib, a COX-2 specific inhibitor, compared with conventional NSAIDs. The SUCCESS trial. *Gastroenterology* **120**, A105.

Goldstein, J. L., Aisenberg, J., Zakko, S. F., Berger, M. F., & Dodge, W. E. (2008). Endoscopic ulcer rates in healthy subjects associated with use of aspirin (81 mg q.D.) alone or coadministered with celecoxib or naproxen: A randomized, 1-week trial. *Digestive Diseases and Sciences* **53**, 647-656.

Gong, Thorn, & Monica (2012). Celecoxib pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenetics and Genomics* **22**, 310-318.

Goodrich et al (2006). Medical treatment of osteoarthritis in the horse – A review. *The Veterinary Journal* **171**, 51-69.

Grave, K., Bangen, M., Engelstad, M., & Soli, N. E. (1992). Prescribing of Veterinary and Human Preparations for Animals in Norway - Was the Preparation Approved for the Animal Species for Which It Was Prescribed. *Journal of Veterinary Pharmacology and Therapeutics* **15**, 45-52.

Guengerich, F. P. (1995). Human cytochrome P-450 enzymes. In *Cytochrome P-450* pp. 473-535.

Guengerich, F. P. (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chemical Research in Toxicology* **14**, 611-650.

Guermouche, M. H. & Gharbi, A. (2004). Simplified solid-phase extraction procedure and liquid chromatographic determination of celecoxib in rat serum. *Chromatographia* **60**, 341-345.

Guirguis, M. S., Sattari, S., & Jamali, F. (2001). Pharmacokinetics of celecoxib in the presence and absence of interferon-induced acute inflammation in the rat: application of a novel HPLC assay. *J.Pharm.Pharm.Sci.* **4**, 1-6.

Gusson, F., Carletti, M., Albo, A. G., Dacasto, M., & Nebbia, C. (2006). Comparison of hydrolytic and conjugative biotransformation pathways in horse, cattle, pig, broiler chick, rabbit and rat liver subcellular fractions. *Veterinary Research Communications* **30**, 271-283.

Hammerschmidt, D. E. (1998). About the cover illustration: The first commercial bottle of aspirin: March 6, 1899. *Journal of Laboratory and Clinical Medicine* **132**, 556.

Harding, A. (2004). Sir John Robert Vane. *The Lancet* **364**, 2090.

Hawkey, C. C., Svoboda, P., Fiedorowicz-Fabrycy, I. F., Nasonov, E. L., Pikhlak, E. G., Cousin, M., Gitton, X., & Hoexter, G. (2004). Gastroduodenal safety and tolerability of

lumiracoxib compared with ibuprofen and celecoxib in patients with osteoarthritis. *Journal of Rheumatology* **31**, 1804-1810.

Hawkey, C. J. (1999). COX-2 inhibitors. *Lancet* **353**, 307-314.

Hegazy, R., Alashhab, M., & Amin, M. (2011). Cardiorenal Effects of Newer NSAIDs (Celecoxib) versus Classic NSAIDs (Ibuprofen) in Patients with Arthritis. *Journal of toxicology* **2011**.

Herbert, C. G. & Johnstone, R. A. W. (2003). *Mass spectrometry basics* CRC Press.

Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., Ng, K. F., Suen, M. W. M., & Tai, H. L. (2003). Electrospray ionisation mass spectrometry: principles and clinical applications. *The Clinical biochemist.Reviews / Australian Association of Clinical Biochemists* **24**, 3-12.

Ho, E. N. M., Kwok, W. H., Leung, D. K. K., Wan, T. S. M., & Wong, A. S. Y. (2007a). Metabolic studies of turinabol in horses. *Analytica Chimica Acta* **586**, 208-216.

Ho, E. N. M., Leung, D. K. K., Wan, T. S. M., & Yu, N. H. (2005). Metabolic studies of methenolone acetate in horses. *Analytica Chimica Acta* **540**, 111-119.

Ho, E. N. M., Leung, D. K. K., Leung, G. N. W., Wan, T. S. M., Wong, H. N. C., Xu, X., & Yeung, J. H. K. (2007b). Metabolic studies of mesterolone in horses. *Analytica Chimica Acta* **596**, 149-155.

Houdeshell, J. W. & Hennessey, P. W. (1977). New Nonsteroidal, Anti-Inflammatory Analgesic for Horses. *Journal of Equine Medicine and Surgery* **1**, 57-63.

Houghton & Maynard (2010). Some Aspects of Doping and Medication Control in Equine Sports. *Handbook of Experimental Pharmacology* **195**, 369-409.

Hwang, S. L., Lee, K. S., Lin, C. L., Lieu, A. S., Cheng, C. Y., Loh, J. K., Hwang, Y. F., Su, Y. F., & Howng, S. L. (2004). Effect of Aspirin and Indomethacin on Prostaglandin E2 Synthesis in C6 Glioma Cells. *The Kaohsiung Journal of Medical Sciences* **20**, 1-4.

Itthipanichpong, C., Chompootaweep, S., Wittayalertpanya, S., Kemsri, W., Thaworn, N., Lilitkarntarakul, P., & Parikamsil, S. (2005). Clinical pharmacokinetic of celecoxib in healthy Thai volunteers. *Journal of the Medical Association of Thailand* **88**, 632-638.

Iwase, M., Kurata, N., Ehana, R., Nishimura, Y., Masamoto, T., & Yasuhara, H. (2006). Evaluation of the effects of hydrophilic organic solvents on CYP3A-mediated drug-drug interaction in vitro. *Human & Experimental Toxicology* **25**, 715-721.

Iyer, L. V., Ho, M. N., Furimsky, A. M., Green, C. E., Green, A. G., Sharp, L. E., Koch, S., Li, Y., Catz, P., Furniss, M., Kapetanovic, I. M., & Nath, S. S. (2004). In vitro metabolism and interaction studies with celecoxib and lovastatin. *AACR Meeting Abstracts* **2004**, 488-48a.

Jackman, B. R., Moore, J. N., Barton, M. H., & Morris, D. D. (1994). Comparison of the Effects of Ketoprofen and Flunixin Meglumine on the In-Vitro Response of Equine Peripheral-Blood Monocytes to Bacterial-Endotoxin. *Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire* **58**, 138-143.

Jaussaud, P., Bellon, C., Besse, S., Courtot, D., & Delatour, P. (1993). Enantioselective Pharmacokinetics of Ketoprofen in Horses. *Journal of Veterinary Pharmacology and Therapeutics* **16**, 373-376.

Jaussaud, P., GUIEU, D., Bellon, C., BARBIER, B., LHOPITAL, M. C., SECHET, R., Courtot, D., & Toutain, P. L. (1992). Pharmacokinetics of tolfenamic acid in the horse. *Equine Veterinary Journal* **24**, 69-72.

Jenkins, W. L. (1987). Pharmacological Aspects of Analgesic Drugs in Animals - An Overview. *Journal of the American Veterinary Medical Association* **191**, 1231-1240.

Jennifer L.Davis, D. P. D. D. ANTI-INFLAMMATORY DRUGS IN THE HORSE. n . 2009.

Ref Type: Magazine Article

Jinsong, N. & Josh, R. (2012). Microdosing Assessment to Evaluate Pharmacokinetics and Drug Metabolism Using Liquid Chromatography-Tandem Mass Spectrometry Technology.

John A Cairns (2007). The coxibs and traditional nonsteroidal anti-inflammatory drugs: A current perspective on cardiovascular risks. *Can J Cardiol Vol 23 No 2 February 2007* **23**, 125-131.

Johnson, C. B., Taylor, P. M., Young, S. S., & Brearley, J. C. (1993). Postoperative Analgesia Using Phenylbutazone, Flunixin Or Carprofen in Horses. *Veterinary Record* **133**, 336-338.

Kallings, P., Johnston, C., & Drevemo, S. (1999b). Effects of flunixin on movement and performance of standardbred trotters on the track. *Equine Vet J Suppl* **30**, 270-273.

Kallings, P., Johnston, C., & Drevemo, S. (1999a). Effects of flunixin on movement and performance of standardbred trotters on the track. *Equine Vet J Suppl* **30**, 270-273.

Kamel, A. & Prakash, C. (2006). High performance liquid chromatography/atmospheric pressure ionization/tandem mass spectrometry (HPLC/API/MS/MS) in drug metabolism and toxicology. *Current Drug Metabolism* **7**, 837-852.

Kargman, S., Charleson, S., Cartwright, M., Frank, J., Riendeau, D., Mancini, J., Evans, J., & O'Neill, G. (1996). Characterization of prostaglandin G/H synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. *Gastroenterology* **111**, 445-454.

Karim, A., Tolbert, D., Burton, E., Piergies, A., Harper, K., Paulson, S., Schoenhard, G., Hubbard, R., Isakson, P., & Geis, S. (1997). SC-58635 (Celecoxib): A highly selective inhibitor of cyclooxygenase-2, disposition kinetics in man and identification of its major CYP450 isozyme in its biotransformation. *Pharmaceutical Research (New York)* **14**, S617.

Kassahun, K., McIntosh, I. S., Shou, M. G., Walsh, D. J., Rodeheffer, C., Slaughter, D. E., Geer, L. A., Halpin, R. A., Agrawal, N., & Rodrigues, A. D. (2001). Role of human liver cytochrome P4503A in the metabolism of etoricoxib, a novel cyclooxygenase-2 selective inhibitor. *Drug Metabolism and Disposition* **29**, 813-820.

Kauffman, G. (1989). Aspirin-Induced Gastric-Mucosal Injury - Lessons Learned from Animal-Models. *Gastroenterology* **96**, 606-614.

Khan, S. A. & Mclean, M. K. (2012). Toxicology of Frequently Encountered Nonsteroidal Anti-Inflammatory Drugs in Dogs and Cats. *Veterinary Clinics of North America-Small Animal Practice* **42**, 289-+.

Kiefer, W. & Dannhardt, G. (2004). Novel insights and therapeutical applications in the field of inhibitors of COX-2. *Curr.Med.Chem.* **11**, 3147-3161.

Kietzmann, M. & Due, M. (2009). What drugs needs the verterinarian at the competition from the pharmacological point of view? Consequences for the reglement using the example of the "Medicine Box". *Pferdeheilkunde* **25**, 322-+.

Kirchheiner, J., Stormer, E., Meisel, C., Steinbach, N., Roots, I., & Brockmoller, J. (2003). Influence of CYP2C9 genetic polymorphisms on pharmacokinetics of celecoxib and its metabolites. *Pharmacogenetics* **13**, 473-480.

Knych, H. K., Corado, C. R., McKemie, D. S., & Steffey, E. P. (2012). Pharmacokinetics and selected pharmacodynamic effects of tramadol following intravenous administration to the horse. *Equine Veterinary Journal* n/a.

Knych, H. K. D., Arthur, R. M., Taylor, A., Moeller, B. C., & Stanley, S. D. (2011). Pharmacokinetics and metabolism of dantrolene in horses. *Journal of Veterinary Pharmacology and Therapeutics* **34**, 238-246.

Knych, H. K. D., Johnson, E. P., Stanley, S. D., & Tell, L. A. (2009). Pharmacokinetics of intravenously administered diclofenac to horses. *Journal of Veterinary Pharmacology and Therapeutics* **32**, 225.

Knych, H. K. D., McKemie, D. S., & Stanley, S. D. (2010). Molecular Cloning, Expression, and Initial Characterization of Members of the CYP3A Family in Horses. *Drug Metabolism and Disposition* **38**, 1820-1827.

Kockaya, E. A., Selmanoglu, G., Kismet, K., & Akay, M. (2010). Pathological and biochemical effects of therapeutic and supratherapeutic doses of celecoxib in Wistar albino male rats. *Drug and Chemical Toxicology* **33**, 410-414.

Koene, M., Goupil, X., Kampmann, C., Hanson, P. D., Denton, D., & Pollmeier, M. G. Field Trial Validation of the Efficacy and Acceptability of Firocoxib, a Highly Selective Cox-2 Inhibitor, in a Group of 96 Lameness Horses. *Journal of Equine Veterinary Science* 30[5], 237-243. 2010.
Ref Type: Abstract

Kopcha, M. & Ahl, A. S. (1989). Experimental Uses of Flunixin Meglumine and Phenylbutazone in Food-Producing Animals. *Journal of the American Veterinary Medical Association* **194**, 45-49.

Krishna, D. R. & Klotz, U. (1994). Extrahepatic Metabolism of Drugs in Humans. *Clinical Pharmacokinetics* **26**, 144-160.

Kruger, K. (2008). [25 years of progress in rheumatology: quantum jumps in the treatment of rheumatoid arthritis]. *MMW Fortschritte der Medizin* **150**.

Kucab, J. E., Lee, C., Chen, C. S., Zhu, J. X., Gilks, C. B., Cheang, M., Huntsman, D., Yorlida, E., Emerman, J., Pollak, M., & Dunn, S. E. (2005). Celecoxib analogues disrupt Akt signaling, which is commonly activated in primary breast tumours. *Breast Cancer Research* **7**, R796-R807.

Kumar & Clark (2009). *Kumar and Clark's Clinical Medicine, 7th Edition*, 2009 ed. Saunders Ltd.

Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., & Stallings, W. C. (1996). Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* **384**, 644-648.

Kvaternick, V., Pollmeier, M., Fischer, J., & Hanson, P. D. (2007). Pharmacokinetics and metabolism of orally administered firocoxib, a novel second generation coxib, in horses. *Journal of Veterinary Pharmacology and Therapeutics* **30**, 208-217.

Laine, L., Curtis, S. P., Cryer, B., Kaur, A., & Cannon, C. P. (2007). Assessment of upper gastrointestinal safety of etoricoxib and diclofenac in patients with osteoarthritis and rheumatoid arthritis in the Multinational Etoricoxib and Diclofenac Arthritis Long-term (MEDAL) programme: a randomised comparison. *Lancet* **369**, 465-473.

Laine, L., Curtis, S. P., Langman, M., Jensen, D. M., Cryer, B., Kaur, A., & Cannon, C. P. (2008). Lower Gastrointestinal Events in a Double-Blind Trial of the Cyclo-Oxygenase-2 Selective Inhibitor Etoricoxib and the Traditional Nonsteroidal Anti-Inflammatory Drug Diclofenac. *Gastroenterology* **135**, 1517-1525.

Laine, L., Wogen, J., & Yu, H. (2003). Gastrointestinal health care resource utilization with chronic use of COX-2-specific inhibitors versus traditional NSAIDs. *Gastroenterology* **125**, 389-395.

Landoni, M. F. & Lees, P. (1995). Comparison of the Antiinflammatory Actions of Flunixin and Ketoprofen in Horses Applying Pk/Pd Modeling. *Equine Veterinary Journal* **27**, 247-256.

Laneuville, O., Breuer, D. K., Dewitt, D. L., Hla, T., Funk, C. D., & Smith, W. L. (1994). Differential Inhibition of Human Prostaglandin Endoperoxide-H Synthase-1 and Synthase-2 by Nonsteroidal Antiinflammatory Drugs. *Journal of Pharmacology and Experimental Therapeutics* **271**, 927-934.

Lascelles, B. D. X., Blikslager, A. T., Fox, S. M., & Reece, D. (2005). Gastrointestinal tract perforation in dogs treated with a selective cyclooxygenase-2 inhibitor: 29 cases (2002-2003). *Javma-Journal of the American Veterinary Medical Association* **227**, 1112-1117.

Lee, C. R., Pieper, J. A., Hinderliter, A. L., Blaisdell, J. A., & Goldstein, J. A. (2002). Evaluation of cytochrome P4502C9 metabolic activity with tolbutamide in CYP2C9*1 heterozygotes. *Clinical Pharmacology & Therapeutics* **72**, 562-571.

Lees, P., Ayliffe, T., Maitho, T. E., & Taylor, J. B. O. (1988). Pharmacokinetics, Metabolism and Excretion of Phenylbutazone in Cattle Following Intravenous, Intramuscular and Oral-Administration. *Research in Veterinary Science* **44**, 57-67.

Lees, P. & Higgins, A. J. (1985). Clinical-Pharmacology and Therapeutic Uses of Non-Steroidal Anti-Inflammatory Drugs in the Horse. *Equine Veterinary Journal* **17**, 83-96.

Lees, P., Maitho, T. E., & Taylor, J. B. (1985). Pharmacokinetics of phenylbutazone in two age groups of ponies: a preliminary study. *Veterinary Record* **116**, 229-232.

Lees, P., May, S. A., Hoeijmakers, M., Coert, A., & Rens, P. V. (1999). A pharmacodynamic and pharmacokinetic study with vedaprofen in an equine model of acute nonimmune inflammation. *Journal of Veterinary Pharmacology and Therapeutics* **22**, 96-106.

Lees, P., Mckellar, Q., May, S. A., & Ludwig, B. (1994b). Pharmacodynamics and Pharmacokinetics of Carprofen in the Horse. *Equine Veterinary Journal* **26**, 203-208.

Lees, P., Mckellar, Q., May, S. A., & Ludwig, B. (1994a). Pharmacodynamics and Pharmacokinetics of Carprofen in the Horse. *Equine Veterinary Journal* **26**, 203-208.

Leese, P. T., Hubbard, R. C., Karim, A., Isakson, P. C., Yu, S. S., & Geis, G. S. (2000). Effects of celecoxib, a novel cyclooxygenase-2 inhibitor, on platelet function in healthy adults: A randomized, controlled trial. *Journal of Clinical Pharmacology* **40**, 124-132.

Letendre, L. T., Tessman, R. K., McClure, S. R., Kvaternick, V. J., Fischer, J. B., & Hanson, P. D. (2008). Pharmacokinetics of firocoxib after administration of multiple consecutive daily doses to horses. *American Journal of Veterinary Research* **69**, 1399-1405.

Leucuta, Laurian, & Vlase (2006). Pharmacokinetics and Metabolic Drug Interactions. *Current Clinical Pharmacology* **1**, 5-20.

Leung, G. N. W., Ho, E. N. M., Leung, D. K. K., Tang, F. P. W., Wan, T. S. M., Yeung, J. H. K., & Wong, H. N. C. (2005). Metabolic studies of clostebol acetate in horses. *Chromatographia* **61**, 397-402.

Levine, W. G. (1978). Biliary-Excretion of Drugs and Other Xenobiotics. *Annual Review of Pharmacology and Toxicology* **18**, 81-96.

Limmroth, V., Katsarava, Z., & Diener, H. C. (1999). Acetylsalicylic Acid in the Treatment of Headache. *Cephalalgia* **19**, 545-551.

Lin, J. H. & Lu, A. Y. H. (1997). Role of Pharmacokinetics and Metabolism in Drug Discovery and Development. *PHARMACOLOGICAL REVIEWS* **49**, 403-449.

Little, D., Brown, S. A., Campbell, N. B., Moeser, A. J., Davis, J. L., & Blikslager, A. T. (2007). Effects of the cyclooxygenase inhibitor meloxicam on recovery of ischemia-injured equine jejunum. *American Journal of Veterinary Research* **68**, 614-624.

Litvak, K. M. & Mcevoy, G. K. (1990). Ketorolac, An Injectable Nonnarcotic Analgesic. *Clinical Pharmacy* **9**, 921-935.

Longo, F., Autefage, A., Bayle, R., Keister, M., & Vangool, F. (1992). Efficacy of A Nonsteroidal Antiinflammatory, Ketofen 10-Percent(R) (Ketoprofen) in the Treatment of Colic in Horses. *Journal of Equine Veterinary Science* **12**, 311-315.

Lutz Brautigam, G. V. I. T. G. H. G. G. (2001). Determination of celecoxib in human plasma and rat microdialysis samples by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B*, **761**, 203-212.

MacAllister, C. G., Morgan, S. J., Borne, A. T., & Pollet, R. A. (1993). Comparison of Adverse-Effects of Phenylbutazone, Flunixin Meglumine, and Ketoprofen in Horses. *Journal of the American Veterinary Medical Association* **202**, 71-77.

Mackay, R. J., French, T. W., Nguyen, H. T., & Mayhew, I. G. (1983). Effects of Large Doses of Phenylbutazone Administration to Horses. *American Journal of Veterinary Research* **44**, 774-780.

Maitho, T. E., Lees, P., & Taylor, J. B. (1986). Absorption and Pharmacokinetics of Phenylbutazone in Welsh Mountain Ponies. *Journal of Veterinary Pharmacology and Therapeutics* **9**, 26-39.

Marnett, L. J. (2009). The COXIB Experience: A Look in the Rearview Mirror. *Annual Review of Pharmacology and Toxicology* **49**, 265-290.

Martineau, H., Thompson, H., & Taylor, D. (2009). Pathology of gastritis and gastric ulceration in the horse. Part 2: A scoring system. *Equine Veterinary Journal* **41**, 646-651.

Matsumoto, A., Melian, A., Shah, A., & Curtis, S. P. (2007). Etoricoxib versus naproxen in patients with rheumatoid arthritis: a prospective, randomized, comparator-controlled 121-week trial. *Curr.Med.Res.Opin.* **23**, 2259-2268.

Matthews, C. Z., Subramanian, R., Woolf, E. J., Foster, N., & Matuszewski, B. K. (2004). Isolation and structural characterization of the photolysis products of etoricoxib. *Pharmazie* **59**, 913-919.

Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry* **75**, 3019-3030.

Maziasz, T., Seibert, K., Khan, N., Paulson, S., & Isakson, P. (1997). Preclinical pharmacology of celecoxib and demonstration of superior GI safety compared with NSAIDs in dogs. *Arthritis and Rheumatism* **40**, 983.

Mccann, M. E., Andersen, D. R., Zhang, D. H., Brideau, C., Black, W. C., Hanson, P. D., & Hickey, G. J. (2004). In vitro effects and in vivo efficacy of a novel cyclooxygenase-2 inhibitor in dogs with experimentally induced synovitis. *American Journal of Veterinary Research* **65**, 503-512.

McConnico, R. S., Morgan, T. W., Williams, C. C., Hubert, J. D., & Moore, R. M. (2008). Pathophysiologic effects of phenylbutazone on the right dorsal colon in horses. *American Journal of Veterinary Research* **69**, 1496-1505.

Mcdowell, M. & Wickler, S. J. (1990). Effect of Naproxen on Bleeding-Time in the Horse. *Journal of Equine Veterinary Science* **10**, 162-163.

Mckellar, Q. A., Bogan, J. A., Vonfellenberg, R. L., Ludwig, B., & Cawley, G. D. (1991). Pharmacokinetic, Biochemical and Tolerance Studies on Carprofen in the Horse. *Equine Veterinary Journal* **23**, 280-284.

Meade, E. A., Smith, W. L., & Dewitt, D. L. (1993). Differential Inhibition of Prostaglandin Endoperoxide Synthase (Cyclooxygenase) Isozymes by Aspirin and Other Nonsteroidal Antiinflammatory Drugs. *Journal of Biological Chemistry* **268**, 6610-6614.

Meek, Mart A.F.J.van de Laar, & Harald E.Vonkeman (2010). Non-Steroidal Anti-Inflammatory Drugs: An Overview of Cardiovascular Risks. *Pharmaceuticals* **3**, 2146-2162.

Merck Sharp & Dohme Limited. Arcoxia 30mg, 60 mg, 90 mg & 120 mg Film-coated Tablets. 2012.

Ref Type: Report

Merlie, J. P., Fagan, D., Mudd, J., & Needleman, P. (1988). Isolation and Characterization of the Complementary-Dna for Sheep Seminal-Vesicle Prostaglandin Endoperoxide Synthase (Cyclooxygenase). *Journal of Biological Chemistry* **263**, 3550-3553.

Merritt, A. M. (1999). Normal equine gastroduodenal secretion and motility. *Equine veterinary journal.Supplement*.

Merskey, H. (1986). Classification of Chronic Pain Descriptions of Chronic Pain Syndromes and Definitions of Pain Terms. *Pain* S1-S226.

Miyamoto, T., Ogino, N., Yamamoto, S., & Hayaishi, O. (1976). Purification of Prostaglandin Endoperoxide Synthetase from Bovine Vesicular Gland Microsomes. *Journal of Biological Chemistry* **251**, 2629-2636.

Modi, S.K.Mody, H.B.Patel, & G.B.Dudhatre (2012). Toxicopathological overview of analgesic and anti-inflammatory drugs in animals. *Journal of Applied Pharmaceutical Science* **2**, 149-157.

Moncada, S., Ferreira, S. H., & Vane, J. R. (1975). Inhibition of Prostaglandin Biosynthesis As Mechanism of Analgesia of Aspirin-Like Drugs in Dog Knee-Joint. *European Journal of Pharmacology* **31**, 250-260.

Morgan, D. B., Carver, M. E., & Payne, R. B. (1977). Plasma Creatinine and Urea - Creatinine Ratio in Patients with Raised Plasma Urea. *British Medical Journal* **2**, 929-932.

Morita, I., Schindler, M., Regier, M. K., Otto, J. C., Hori, T., Dewitt, D. L., & Smith, W. L. (1995). Different Intracellular Locations for Prostaglandin Endoperoxide-H Synthase-1 and Synthase-2. *Journal of Biological Chemistry* **270**, 10902-10908.

Morris, M. C., Allanby, C. W., Toseland, P., Haycock, G. B., Chantler, C., & Barratt, M. (1982). Evaluation of A Height Plasma Creatinine Formula in the Measurement of Glomerular-Filtration Rate. *Archives of Disease in Childhood* **57**, 611-615.

Moses, V. S. & Bertone, A. L. (2002). Nonsteroidal anti-inflammatory drugs. *Veterinary Clinics of North America-Equine Practice* **18**, 21-+.

Mroszczak, E. J., Jung, D., Yee, J., Bynum, L., Sevelius, H., & Massey, I. (1990). Ketorolac Tromethamine Pharmacokinetics and Metabolism After Intravenous, Intramuscular, and Oral-Administration in Humans and Animals. *Pharmacotherapy* **10**, S33-S39.

- Nakahura, T., Griswold, W., Lemire, J., Mendoza, S., & Reznik, V. (1998). Nonsteroidal anti-inflammatory drug use in adolescence. *Journal of Adolescent Health* **23**, 307-310.
- Nebbia, C., Dacasto, M., Giaccherino, A. R., Albo, A. G., & Carletti, M. (2003). Comparative expression of liver cytochrome P450-dependent monooxygenases in the horse and in other agricultural and laboratory species. *Veterinary Journal* **165**, 53-64.
- Nelson, D. R. (2013). A world of cytochrome P450s. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368**.
- Nicoll-Griffith, D. A., Falgoutyret, J. P., Silva, J. M., Morin, P. E., Trimble, L., Chan, C. C., Clas, S., Leger, S., Wang, Z. Y., Yergey, J. A., & Riendeau, D. (1999). Oxidative bioactivation of the lactol prodrug of a lactone cyclooxygenase-2 inhibitor. *Drug Metabolism and Disposition* **27**, 403-409.
- Nikonova, L., V (1998). To the justification of the effect of diuciphone electrophoresis in dimethylsulfoxide on the microhemocirculation and peripheric blood flow in complex therapy of the Bekhterev disease. *Kazanskii Meditsinskii Zhurnal* **79**, 436-441.
- Nishiya, Y., Nakamura, K., Okudaira, N., Abe, K., Kobayashi, N., & Okazaki, O. (2010). Effects of organic solvents on the time-dependent inhibition of CYP3A4 by diazepam. *Xenobiotica* **40**, 1-8.
- Noble, S. L., King, D. S., & Olutade, J. I. (2000). Cyclooxygenase-2 enzyme inhibitors: place in therapy. *Am.Fam.Physician* **61**, 3669-3676.
- Nuno Martinho, C. D. C. P. R. (2011). Recent Advances in Drug Delivery Systems. *Journal of Biomaterials and Nanobiotechnology* **2**, 510-526.
- Nussmeier, N. A., Whelton, A. A., Brown, M. T., Langford, R. M., Hoeft, A., Parlow, J. L., Boyce, S. W., & Verburg, K. M. (2005). Complications of the COX-2 inhibitors parecoxib and valdecoxib after cardiac surgery. *N Engl J Med* **352**, 1081-1091.
- Obach, R. S., Baxter, J. G., Liston, T. E., Silber, B. M., Jones, B. C., MacIntyre, F., Rance, D. J., & Wastall, P. (1997). The prediction of human pharmacokinetic parameters from preclinical and in vitro metabolism data. *Journal of Pharmacology and Experimental Therapeutics* **283**, 46-58.
- Olavi Pelkonen (2009). Comparison of Metabolic Stability and Metabolite Identification of 55 ECVAM/ICCVAM Validation Compounds between Human and

Rat Liver Homogenates and Microsomes? - A Preliminary Analysis. *Alternatives to Animal Experimentation (ALTEX)*, **26** 214-222, 214-222.

Omura, T. & Sato, R. (1964). The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J Biol Chem* **239**.

Orsini, J. A., Ryan, W. G., Carithers, D. S., & Boston, R. C. (2012). Evaluation of oral administration of firocoxib for the management of musculoskeletal pain and lameness associated with osteoarthritis in horses. *American Journal of Veterinary Research* **73**, 664-671.

Otto, K. A. & Short, C. E. (1998). Pharmaceutical control of pain in large animals. *Applied Animal Behaviour Science* **59**, 157-169.

Patterson, R., Bello, A. E., & Lefkowitz, J. (1999). Immunologic tolerability profile of celecoxib. *Clinical Therapeutics* **21**, 2065-2079.

Paulekas, R. & Haussler, K. K. (2009). Principles and Practice of Therapeutic Exercise for Horses. *Journal of Equine Veterinary Science* **29**, 870-893.

Paulson, S. K., Hribar, J. D., Liu, N. W. K., Hajdu, E., Bible, R. H., Piergies, A., & Karim, A. (2000a). Metabolism and excretion of [C-14]celecoxib in healthy male volunteers. *Drug Metabolism and Disposition* **28**, 308-314.

Paulson, S. K., Vaughn, M. B., Jessen, S. M., Lawal, Y., Gresk, C. J., Yan, B., Maziasz, T. J., Cook, C. S., & Karim, A. (2001). Pharmacokinetics of celecoxib after oral administration in dogs and humans: Effect of food and site of absorption. *Journal of Pharmacology and Experimental Therapeutics* **297**, 638-645.

Paulson, S. K., Zhang, J. Y., Jessen, S. M., Lawal, Y., Liu, N. W. K., Dudkowski, C. M., Wang, Y. F., Chang, M., Yang, D., Findlay, J. W. A., Berge, M. A., Markos, C. S., Breau, A. P., Hribar, J. D., & Yuan, J. (2000b). Comparison of celecoxib metabolism and excretion in mouse, rabbit, dog, cynomolgus monkey and rhesus monkey. *Xenobiotica* **30**, 731-744.

Peloso, P. M., Gammaitoni, A., Smugar, S. S., Wang, H. W., & Moore, A. R. (2011). Longitudinal Numbers-Needed-To-Treat (NNT) for Achieving Various Levels of Analgesic Response and Improvement with Etoricoxib, Naproxen, and Placebo in Ankylosing Spondylitis. *Bmc Musculoskeletal Disorders* **12**.

Perrone, R. D., Madias, N. E., & Levey, A. S. (1992). Serum Creatinine As An Index of Renal-Function - New Insights Into Old Concepts. *Clinical Chemistry* **38**, 1933-1953.

Peters, L. M., Demmel, S., Pusch, G., Buters, J. T. M., Thormann, W., Zielinski, J., Leeb, T., Mevissen, M., & Schmitz, A. (2013). Equine cytochrome P450 2B6--genomic identification, expression and functional characterization with ketamine. *Toxicology and Applied Pharmacology* **266**.

Pfizer Ltd. Celebrex (celecoxib): summary of product characteristics [online]. Available from URL:

<http://www.medicines.org.uk/EMC/medicine/14534/SPC/Celebrex+100mg+200mg+capsules>. 2012.

Ref Type: Report

Poulin, P. & Haddad, S. (2012). Advancing prediction of tissue distribution and volume of distribution of highly lipophilic compounds from a simplified tissue-composition-based model as a mechanistic animal alternative method. *Journal of Pharmaceutical Sciences* **101**, 2250-2261.

Powis, G. (1989). The Use of Human-Liver for Foreign Compound Metabolism and Toxicity Studies. *Drug Metabolism Reviews* **20**, 379-394.

Prakash, C., Shaffer, C. L., & Nedderman, A. (2007). Analytical strategies for identifying drug metabolites. *Mass Spectrometry Reviews* **26**, 340-369.

Prouse, P. J., Bevis, P. J., Bluhmki, E., & Distel, M. (2005). Evaluation of the safety, tolerability, and efficacy of meloxicam tablets in patients with osteoarthritis. *Clinical Therapeutics* **18**, 429-439.

Radwan, M. A., Baky, N. A. A., Zaghloul, I., & Aboul-Enein, H. Y. (2012). Pharmacokinetics and cardiovascular effect of etoricoxib in the absence or presence of St. John's Wort in rats. *Arzneimittel-Forschung* **62**, 313-318.

Radwan, M. A., Zaghloul, I. Y., & Abd Elbaky, N. A. (2009). Stability indicating high performance liquid chromatographic assay for the pharmacokinetics of cyclooxygenase (COX-2) inhibitor etoricoxib in rats. *African Journal of Pharmacy and Pharmacology* **3**, 339-346.

Rahme, E., Marentette, M. A., Kong, S. X., & LeLorier, J. (2002). Use of NSAIDs, COX-2 inhibitors, and acetaminophen and associated coprescriptions of gastroprotective agents in an elderly population. *Arthritis & Rheumatism-Arthritis Care & Research* **47**, 595-602.

Ramey, D. R., Watson, D. J., Yu, C., Bolognese, J. A., Curtis, S. P., & Reicin, A. S. (2005). The incidence of upper gastrointestinal adverse events in clinical trials of

etoricoxib vs. non-selective NSAIDs: an updated combined analysis. *Curr.Med.Res.Opin.* **21**, 715-722.

Rane, A., Wilkinson, G. R., & Shand, D. G. (1977). Prediction of Hepatic Extraction Ratio from Invitro Measurement of Intrinsic Clearance. *Journal of Pharmacology and Experimental Therapeutics* **200**, 420-424.

Rao, R. N., Meena, S., Nagaraju, A., Rao, A. R., & Ravikanth, S. (2006). Liquid-chromatographic separation and determination of process-related impurities, including a regio-specific isomer of celecoxib on reversed-phase C18 column dynamically coated with hexamethyldisilazane. *Analytical Sciences* **22**, 1257-1260.

Raskin, J. B. (1999). Gastrointestinal effects of nonsteroidal anti-inflammatory therapy. *The American Journal of Medicine* **106**, 3S-12S.

Remmer, H. (1970). Role of Liver in Drug Metabolism. *American Journal of Medicine* **49**, 617-&.

Riendeau, D., Percival, M. D., Brideau, C., Charleson, S., Dube, D., Ethier, D., Falgueyret, J. P., Friesen, R. W., Gordon, R., Greig, G., Guay, J., Mancini, J., Ouellet, M., Wong, E., Xu, L., Boyce, S., Visco, D., Girard, Y., Prasit, P., Zamboni, R., Rodger, I. W., Gresser, M., Ford-Hutchinson, A. W., Young, R. N., & Chan, C. C. (2001). Etoricoxib (MK-0663): preclinical profile and comparison with other agents that selectively inhibit cyclooxygenase-2. *J.Pharmacol.Exp.Ther.* **296**, 558-566.

Rifkin, B. & Perazella, M. (2005). Analgesic Therapy in Patients with Chronic Kidney Disease: A Case-Based Approach. *Hospital Physician* **43**, 13-22.

Rodrigues, A. D., Halpin, R. A., Geer, L. A., Cui, D. H., Woolf, E. J., Matthews, C. Z., Gottesdiener, K. M., Larson, P. J., Lasseter, K. C., & Agrawal, N. G. B. (2003). Absorption, metabolism, and excretion of etoricoxib, a potent and selective cyclooxygenase-2 inhibitor, in healthy male volunteers. *Drug Metabolism and Disposition* **31**, 224-232.

Rohner K & Demuth D (1994). Adverse effects of veterinary drugs. *Schweiz Arch Tierheilkd.* **136**, 309-312.

Rose, M. J., Woolf, E. J., & Matuszewski, B. K. (2000). Determination of celecoxib in human plasma by normal-phase high-performance liquid chromatography with column switching and ultraviolet absorbance detection. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **738**, 377-385.

Routledge & Shand (1979). Presystemic Drug Elimination. *Annual Review of Pharmacology and Toxicology* **19**, 447-468.

Ruschitzka (2007). Painful lessons: COX-2 inhibitors, NSAIDs, and hypertension. *Current Hypertension Reports* **9**, 41-44.

Ruschitzka, F. T. (2005). The APPROVe study: What we should learn from the VIOXX withdrawal. *Current Hypertension Reports* **7**, 41-43.

Samuelsson, B., Morgenstern, R., & Jakobsson, P. J. (2007). Membrane Prostaglandin E Synthase-1: A Novel Therapeutic Target. *PHARMACOLOGICAL REVIEWS* **59**, 207-224.

Sandberg, M., Yasar, U., Stromberg, P., Hoog, J. O., & Eliasson, E. (2002). Oxidation of celecoxib by polymorphic cytochrome P4502C9 and alcohol dehydrogenase. *British Journal of Clinical Pharmacology* **54**, 423-429.

Sandhya Sanghi & Eric J. MacLaughlin, C. W. J. S. C. P. J. N. L. W. a. D. E. D. (2006). Cyclooxygenase-2 Inhibitors: A Painful Lesson. *Cardiovascular & Haematological Disorders-Drug Targets* **6**, 83-98.

Scarth, J. P., Spencer, H. A., Hudson, S. C., Teale, P., Gray, B. P., & Hillyer, L. L. (2010a). The application of in vitro technologies to study the metabolism of the androgenic/anabolic steroid stanozolol in the equine. *Steroids* **75**, 57-69.

Scarth, J. P., Spencer, H. A., Timbers, S. E., Hudson, S. C., & Hillyer, L. L. (2010b). The use of in vitro technologies coupled with high resolution accurate mass LC-MS for studying drug metabolism in equine drug surveillance. *Drug Testing and Analysis* **2**, 1-10.

Scheiman, J. M., Yeomans, N. D., Talley, N. J., Vakil, N., Chan, F. K. L., Tulassay, Z., Ralnoldi, J. L., Szczepanski, L., Ung, J. A., Kleczkowski, D., Ahlbom, H., Naesdal, J., & Hawkey, C. (2006). Prevention of ulcers by esomeprazole in at-risk patients using non-selective NSAIDs and COX-2 inhibitors. *American Journal of Gastroenterology* **101**, 701-710.

Scher, J. U. & Pillinger, M. H. (2009). The Anti-Inflammatory Effects of Prostaglandins. *Journal of Investigative Medicine* **57**, 703-708.

Scheubel Emmanuel. Predictive in vitro dissolution tools: Application during formulation development. 2010. Faculty of Pharmacy, University Clermont-Ferrand 1, France.

Ref Type: Thesis/Dissertation

Schwartz, J. I., Thach, C., Lasseter, K. C., Miller, J., Hreniuk, D., Hilliard, D. A., Snyder, K. M., Gertz, B. J., & Gottesdiener, K. M. (2007). Effects of etoricoxib and comparator nonsteroidal anti-inflammatory drugs on urinary sodium excretion, blood pressure, and other renal function indicators in elderly subjects consuming a controlled sodium diet. *J.Clin.Pharmacol.* **47**, 1521-1531.

Schwartz, J. L., Dallob, A. L., Larson, P. J., Laterza, O. E., Miller, J., Royalty, J., Snyder, K. M., Chappell, D. L., Hilliard, D. A., Flynn, M. E., Cavanaugh, P. F., & Wagner, J. A. (2008). Comparative inhibitory activity of etoricoxib, celecoxib, and diclofenac on COX-2 versus COX-1 in healthy subjects. *Journal of Clinical Pharmacology* **48**, 745-754.

Scott, L. J. & Perry, C. M. (2000). Tramadol - A review of its use in perioperative pain. *Drugs* **60**, 139-176.

Searle. Celebrex (celecoxib) capsules: US prescribing information (online). Available from URL:http://www.pfizer.com/files/products/uspi_celebrex.pdf [Accessed 2011 Feb 17]. 2011.

Ref Type: Internet Communication

Seibert, K. & Masferrer, J. L. (1994). Role of Inducible Cyclooxygenase (Cox-2) in Inflammation. *Receptor* **4**, 17-23.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., & Isakson, P. (1997). *Distribution of COX-1 and COX-2 in normal and inflamed tissues*, pp. 167-170. PLENUM PRESS DIV PLENUM PUBLISHING CORP, NEW YORK.

Shakya, A. K. & Khalaft, N. A. (2007). High performance liquid chromatographic and ultra violet spectroscopic determination of etoricoxib in pharmaceutical formulations. *Asian Journal of Chemistry* **19**, 2059-2069.

Shannon K.Reed, Nathaniel T.Messer, Ronald K.Tessman, & Kevin G.Keegan (2006). Effects of phenylbutazone alone or in combination with flunixin meglumine on blood protein concentrations in horses. *American Journal of Veterinary Research* **67**, 398-402.

Shi, S. & Klotz, U. (2008). Clinical use and pharmacological properties of selective COX-2 inhibitors. *Eur.J.Clin.Pharmacol.* **64**, 233-252.

Shilo, Y., Britzi, M., Eytan, B., Lifschitz, T., Soback, S., & Steinman, A. (2008). Pharmacokinetics of tramadol in horses after intravenous, intramuscular and oral administration. *Journal of Veterinary Pharmacology and Therapeutics* **31**, 60-65.

Short, C. E. (1995). Equine Pain - Use of Nonsteroidal Antiinflammatory Drugs and Analgesics for Its Prevention and Control. *Equine Practice* **17**, 12-22.

Silverstein, F. E., Faich, G., Goldstein, J. L., Simons, L. S., Pincus, T., Whelton, A., Makuch, R., Eisen, G., Agrawal, N. M., & Stenson, W. F. (2000a). Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* **284**, 1247-1255.

Silverstein, F. E., Faich, G., Goldstein, J. L., Simon, L. S., Pincus, T., Whelton, A., Makuch, R., Eisen, G., Agrawal, N. M., Stenson, W. F., Burr, A. M., Zhao, W. W., Kent, J. D., Lefkowitz, J. B., Verburg, K. M., & Geis, G. S. (2000b). Gastrointestinal Toxicity With Celecoxib vs Nonsteroidal Anti-inflammatory Drugs for Osteoarthritis and Rheumatoid Arthritis: The CLASS Study: A Randomized Controlled Trial. *JAMA: The Journal of the American Medical Association* **284**, 1247-1255.

Simmons, D. L., Chandrasekharan, N. V., Hu, D., Roos, K. L., & Tomsik, J. (2005). Comments on "Acetaminophen and the cyclooxygenase-3 puzzle: Sorting out facts, fictions, and uncertainties". *Journal of Pharmacology and Experimental Therapeutics* **315**, 1412-1414.

Simon, L. S., Lanza, F. L., Lipsky, P. E., Hubbard, R. C., Talwalker, S., Schwartz, B. D., Isakson, P. C., & Geis, G. S. (1998). Preliminary study of the safety and efficacy of SC-58635, a novel cyclooxygenase 2 inhibitor - Efficacy and safety in two placebo-controlled trials in osteoarthritis and rheumatoid arthritis, and studies of gastrointestinal and platelet effects. *Arthritis and Rheumatism* **41**, 1591-1602.

Singh, G., Goldstein, J., Bensen, W., Agrawal, N., Eisen, G., Fort, J., Bello, A., & Boots, S. (2001). SUCCESS-1 in osteoarthritis (OA) trial: Celecoxib vs NSAIDs and risk of serious upper GI (UGI) complications. *Journal of Rheumatology* **28**, 5.

Singh, V. P., Patil, C. S., & Kulkarni, S. K. (2006). Analysis of interaction between etoricoxib and tramadol against mechanical hyperalgesia of spinal cord injury in rats. *Life Sci.* **78**, 1168-1174.

Skoog, D. A. & Leary, J. J. (1992). Mass Spectrometry. In Principles of Instrumental Analysis, 4th ed.; Saunders College Publishing: Orlando, FL, U.S.A.. pp. 420-461.

Slaughter, R. L. & Edwards, D. J. (1995). Recent advances: the cytochrome P450 enzymes. *The Annals of Pharmacotherapy* **29**, 619-624.

Slingsby, L. S., Taylor, P. M., & Waterman-Pearson, A. E. (2006). Effects of two doses of buprenorphine four or six hours apart on nociceptive thresholds, pain and sedation in does after castration. *Veterinary Record* **159**, 705-711.

Smith, W. L., Dewitt, D. L., & Garavito, R. M. (2000). Cyclooxygenases: Structural, cellular, and molecular biology. *Annual Review of Biochemistry* **69**, 145-182.

Smith, W. L. & Marnett, L. J. (1991). Prostaglandin endoperoxide synthase: structure and catalysis. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1083**, 1-17.

Snow, D. (1981). Non-steroidal anti-inflammatory agents in the horse. *In Pract* **3**, 24-8, 30.

Srinivasu, M., Narayana, C., Rao, D., Sr., & Reddy, G. (2000). A validated LC method for the quantitative determination of celecoxib in pharmaceutical dosage forms and purity evaluation in bulk drugs. *Journal of Pharmaceutical and Biomedical Analysis* **22**, 949-956.

Staack, R. F. & Hopfgartner, G. (2007). New analytical strategies in studying drug metabolism. *Analytical and Bioanalytical Chemistry* **388**, 1365-1380.

Stelio P.L.Luna, Ana C.Basilio, Paulo V.M.Steagall, Luciana P.Machado, & Flávia Q.Moutinho (2007). Evaluation of adverse effects of long-term oral administration of carprofen, etodolac, flunixin meglumine, ketoprofen, and meloxicam in dogs. *American Journal of Veterinary Research* **68**, 258-264.

Stempak, D., Gammon, J., Halton, J., Champagne, M., Koren, G., & Baruchel, S. (2005). Modulation of celecoxib pharmacokinetics by food in pediatric patients. *Clin.Pharmacol.Ther.* **77**, 226-228.

Stevenson, D. D. (1984). Diagnosis, Prevention, and Treatment of Adverse Reactions to Aspirin and Nonsteroidal Anti-Inflammatory Drugs. *Journal of Allergy and Clinical Immunology* **74**, 617-622.

Stewart, A. J., Boothe, D. M., Cruz-Espindola, C., Baird, E., & Springfield, J. (2008). Pharmacokinetics and urine detection of tramadol in adult horses. *Journal of Veterinary Internal Medicine* **22**, 393.

Stormer, E., Bauer, S., Kirchheiner, J., Brockmoller, J., & Roots, I. (2003). Simultaneous determination of celecoxib, hydroxycelecoxib, and carboxycelecoxib in human plasma using gradient reversed-phase liquid chromatography with ultraviolet absorbance detection. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **783**, 207-212.

Sturmer, T., Erb, A., Keller, F., Gunther, K. P., & Brenner, H. (2001). Determinants of impaired renal function with use of nonsteroidal anti-inflammatory drugs: The importance of half-life and other medications. *American Journal of Medicine* **111**, 521-527.

Sumida, K., Igarashi, Y., Toritsuka, N., Matsushita, T., Abe-Tomizawa, K., Aoki, M., Urushidani, T., Yamada, H., & Ohno, Y. (2011). Effects of DMSO on gene expression in human and rat hepatocytes. *Human & Experimental Toxicology* **30**, 1701-1709.

Sun, F. F., Chapman, J. P., & McGuire, J. C. (1977). Metabolism of Prostaglandin Endoperoxide in Animal-Tissues. *Prostaglandins* **14**, 1055-1074.

Tacconelli, S., Capone, M. L., & Patrignani, P. (2004). Clinical pharmacology of novel selective COX-2 inhibitors. *Current Pharmaceutical Design* **10**, 589-601.

Tang, C., Shou, M., Rushmore, T. H., Mei, Q., Sandhu, P., Woolf, E. J., Rose, M. J., Gelmann, A., Greenberg, H. E., De, L., I, Van Hecken, A., De Schepper, P. J., Ebel, D. L., Schwartz, J. I., & Rodrigues, A. D. (2001). In-vitro metabolism of celecoxib, a cyclooxygenase-2 inhibitor, by allelic variant forms of human liver microsomal cytochrome P450 2C9: correlation with CYP2C9 genotype and in-vivo pharmacokinetics. *Pharmacogenetics* **11**, 223-235.

Tang, C. Y., Shou, M. G., Mei, Q., Rushmore, T. H., & Rodrigues, A. D. (2000). Major role of human liver microsomal cytochrome p450 2C9 (CYP2C9) in the oxidative metabolism of celecoxib, a novel cyclooxygenase-II inhibitor. *Journal of Pharmacology and Experimental Therapeutics* **293**, 453-459.

Teale & Houghton (2010). Metabolism of anabolic steroids and their relevance to drug detection in horseracing. *Bioanalysis* **2**, 1085-1107.

Thiefin (2005). Toxic effects of nonsteroidal antiinflammatory drugs on the small bowel, colon, and rectum. *Joint Bone Spine* **72**, 286-294.

Thomas P.Colville (2002). *Clinical Anatomy and Physiology for Veterinary Technicians*, 2nd edition ed. MOSBY Elsevier.

Thummel & Wilkinson (1998). IN VITRO AND IN VIVO DRUG INTERACTIONS INVOLVING HUMAN CYP3A. *Annu.Rev.Pharmacol.Toxicol.* **38**, 389-430.

Thurman, E. M. & Ferrer, I. (2002). Comparison of quadrupole-time-of-flight (Q-TOF), triple quadrupole, and ion-trap mass spectrometry/mass spectrometry (MS/MS) for the analysis of unknown pesticides and pharmaceuticals in water. *Abstracts of Papers American Chemical Society* **223**, 10.

Tobin, T. (1979). Pharmacology Review - Non-Steroidal Anti-Inflammatory Drugs .2. Equiproxen, Meclofenamic Acid, Flunixin and Others. *Journal of Equine Medicine and Surgery* **3**, 298-302.

Tobin, T., Chay, S., Kamerling, S., Woods, W. E., Weckman, T. J., Blake, J. W., & Lees, P. (1986). Phenylbutazone in the Horse - A Review. *Journal of Veterinary Pharmacology and Therapeutics* **9**, 1-25.

Toutain, P. L. & Bousquet-Melou, A. (2004a). Bioavailability and its assessment. *Journal of Veterinary Pharmacology and Therapeutics* **27**, 455-466.

Toutain, P. L. & Bousquet-Melou, A. (2004b). Plasma terminal half-life. *Journal of Veterinary Pharmacology and Therapeutics* **27**, 427-439.

Turck, D., Busch, U., Heinzl, G., & Narjes, H. (1997). Clinical pharmacokinetics of meloxicam. *Arzneimittel-Forschung/Drug Research* **47**, 253-258.

Turner, Sean, & •Violet Ford (2004). Role of the selective cyclo-oxygenase-2 (COX-2) inhibitors in children. *Arch Dis Child Educ Pract Ed* **89**, 46-49.

Tyden, E., Olsen, L., Tallkvist, J., Larsson, P., & Tjalve, H. (2004). CYP3A in horse intestines. *Toxicology and Applied Pharmacology* **201**, 112-119.

URSO, BLARD, & GIORGI (2002). A short introduction to pharmacokinetics. *European Review for Medical and Pharmacological Sciences* **6**, 33-44.

Vadnerkar, G., Jain, S., & Jain, D. (2006). Validated bicanalytical method development for determination of etoricoxib in human plasma and its application to pharmacokinetic studies in rats. *Journal of Pharmacy and Pharmacology* **58**, A5-A6.

Van der Bijl, P. & Van der Bul, P. (2003). Efficacy, safety and potential clinical roles of the COX-2-specific inhibitors. *International Journal of Immunopathology and Pharmacology* **16**, 17-22.

Vane, J. (1994). Pharmacology - Towards A Better Aspirin. *Nature* **367**, 215-216.

Vane, J. R. (2000). The fight against rheumatism: From willow bark to COX-1 sparing drugs. *Journal of Physiology and Pharmacology* **51**, 573-586.

Vane, J. R., Bakhle, Y. S., & Botting, R. M. (1998). Cyclooxygenases 1 and 2. *Annual Review of Pharmacology and Toxicology* **38**, 97-120.

Vane, J. R. & Botting, R. M. (1998a). Anti-inflammatory drugs and their mechanism of action. *Inflammation Research* **47**, S78-S87.

Vane, J. R. & Botting, R. M. (1998b). Mechanism of action of antiinflammatory drugs. *International Journal of Tissue Reactions-Experimental and Clinical Aspects* **20**, 3-15.

Vane, J. R. & Botting, R. M. (2000). The future of NSAID therapy: selective COX-2 inhibitors. *International Journal of Clinical Practice* **54**, 7-9.

Vane, J. R. & Botting, R. M. (2003). The mechanism of action of aspirin. *Thrombosis Research* **110**, 255-258.

Vigdahl, R. L. & Tukey, R. H. (1977). Mechanism of action of novel anti-inflammatory drugs diflumidone and R-805. *Biochemical Pharmacology* **26**, 307-311.

Villa, R., Cagnardi, P., Belloli, C., Zonca, A., Zizzadoro, C., Ferro, E., & Carli, S. (2007). Oral and intravenous administration of nimesulide in the horse: rational dosage regimen from pharmacokinetic and pharmacodynamic data. *Equine Veterinary Journal* **39**, 136-142.

Voneuler, U. S. (1983). History and Development of Prostaglandins. *General Pharmacology-the Vascular System* **14**, 3-6.

Vonkeman, M. P. *. a. A. F. J. v. d. L. M. P. (2008). Nonsteroidal Anti-Inflammatory Drugs: Adverse Effects and Their Prevention. *Semin Arthritis Rheum*.

Wang, J. F. & Chou, K. C. (2010). Molecular Modeling of Cytochrome P450 and Drug Metabolism. *Current Drug Metabolism* **11**, 342-346.

Warden, S. J. (2005). Cyclo-oxygenase-2 inhibitors : beneficial or detrimental for athletes with acute musculoskeletal injuries? *Sports Med.* **35**, 271-283.

Watkins, P. B., Kaplowitz, N., Slattery, J. T., Colonese, C. R., Colucci, S. V., Stewart, P. W., & Harris, S. C. (2006). Aminotransferase elevations in healthy adults receiving 4 grams of acetaminophen daily - A randomized controlled trial. *Jama-Journal of the American Medical Association* **296**, 87-93.

Watson, D., Walesby, H., Barker, S., & Short, C. (2004). In vitro distribution and elimination of 20% phenylbutazone solution administered orally to fasted horses. *Journal of Veterinary Internal Medicine* **18**, 459.

Werner, U., Werner, D., Pahl, A., Mundkowski, R., Gillich, M., & Brune, K. (2002). Investigation of the pharmacokinetics of celecoxib by liquid chromatography-mass spectrometry. *Biomed.Chromatogr.* **16**, 56-60.

Whelton, A., Stout, R. L., Spilman, P. S., & Klassen, D. K. (1990). Renal effects of ibuprofen, piroxicam, and sulindac in patients with asymptomatic renal failure. A prospective, randomized, crossover comparison. *Annals Of Internal Medicine* **112**, 568-576.

Whelton, A. (2001). Renal aspects of treatment with conventional nonsteroidal anti-inflammatory drugs versus cyclooxygenase-2 specific inhibitors. *The American Journal of Medicine* **110**, 33-42.

Wilhelmi, B., Naicker, K., & De Kock, S. (2009). In vitro and in vivo analysis of drug metabolism in liver microsomes and from a horse administration trial. *Febs Journal* **276**, 185.

William L.Smith, D. L. D. a. R. M. G. (2000). CYCLOOXYGENASES: Structural, Cellular, and Molecular Biology. *Annu.Rev.Biochem.* **69**, 145-182.

Willoughby, D. A., Moore, A. R., & Colville-Nash, P. R. (2000). COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease. *Lancet* **355**, 646-648.

Wilson, A. W., Medhurst, S. J., Dixon, C. I., Bontoft, N. C., Winyard, L. A., Brackenborough, K. T., De Alba, J., Clarke, C. J., Gunthorpe, M. J., Hicks, G. A., Bountra, C., McQueen, D. S., & Chessell, I. P. (2006). An animal model of chronic inflammatory pain: pharmacological and temporal differentiation from acute models. *Eur.J.Pain* **10**, 537-549.

Wong, J. K. Y., Tang, F. P. W., & Wan, T. S. M. (2011). In vitro metabolic studies using homogenized horse liver in place of horse liver microsomes. *Drug Testing and Analysis* **3**, 393-399.

Wrighton, S. A., Vandenbranden, M., Stevens, J. C., Shipley, L. A., Ring, B. J., Rettie, A. E., & Cashman, J. R. (1993). In-Vitro Methods for Assessing Human Hepatic Drug-Metabolism - Their Use in Drug Development. *Drug Metabolism Reviews* **25**, 453-484.

Wu, K. K. & Liou, J. Y. (2005). Cellular and molecular biology of prostacyclin synthase. *Biochemical and Biophysical Research Communications* **338**, 45-52.

Wu, W. N., Mckown, L. A., Gauthier, A. D., Jones, W. J., & Raffa, R. B. (2001). Metabolism of the analgesic drug, tramadol hydrochloride, in rat and dog. *Xenobiotica* **31**, 423-441.

Yokoyama, C., Takai, T., & Tanabe, T. (1988). Primary Structure of Sheep Prostaglandin Endoperoxide Synthase Deduced from Cdna Sequence. *FEBS Letters* **231**, 347-351.

Yoshino, T., Kimoto, A., Kobayashi, S., Noguchi, M., Fukunaga, M., Hayashi, A., Miyata, K., & Sasamata, M. (2005). Pharmacological profile of celecoxib, a specific cyclooxygenase-2 inhibitor. *Arzneimittel-Forschung-Drug Research* **55**, 394-402.

Yost, R. A. & Enke, C. G. (1978). Selected Ion Fragmentation with A Tandem Quadrupole Mass-Spectrometer. *Journal of the American Chemical Society* **100**, 2274-2275.

Young & Yeow. Detection of naproxen and its metabolites in horses. Proceedings of the Fifth International Conference on the Control of the Use of Drugs in Racehorses (Toronto)[The Canadian Section Association of Official Racing Chemists: Toronto, Canada.], 200-207. 1983.

Ref Type: Conference Proceeding

Yuan, R., Madani, S., Wei, X. X., Reynolds, K., & Huang, S. M. (2002). Evaluation of Cytochrome P450 Probe Substrates Commonly Used by the Pharmaceutical Industry to Study in Vitro Drug Interactions. *Drug Metabolism and Disposition* **30**, 1311-1319.

Yuan, Y. H. & Hunt, R. H. (2007). Global gastrointestinal safety profile of etoricoxib and lumiracoxib. *Current Pharmaceutical Design* **13**, 2237-2247.

Zapata, G. L., Britos, R. M., Pintos, M. E., Dreizzen, E., Lausada, N. I. B., & Arauz, S. (2005). Tear urea nitrogen and creatinine levels in horse and their correlation with serum values. *Veterinary Ophthalmology* **8**, 207-209.

Zarghi, A. & Arfaei, S. (2011). Selective COX-2 Inhibitors: A Review of Their Structure-Activity Relationships. *Iranian Journal of Pharmaceutical Research* **10**, 655-683.

Zarghi, A., Shafaati, A., Foroutan, S. M., & Khoddam, A. (2006a). Simple and rapid high-performance liquid chromatographic method for determination of celecoxib in plasma using UV detection: Application in pharmacokinetic studies. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **835**, 100-104.

Zarghi, A., Shafaati, A., Foroutan, S. M., & Khoddam, A. (2006b). Simple and rapid high-performance liquid chromatographic method for determination of celecoxib in plasma using UV detection: application in pharmacokinetic studies. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.* **835**, 100-104.

Zarraga, I. G. E. & Schwarz, E. R. (2007). Coxibs and heart disease - What we have learned and what else we need to know. *Journal of the American College of Cardiology* **49**, 1-14.

Zhang, J. Y., Wang, Y., Dudkowski, C., Yang, D. C., Chang, M., Yuan, J. H., Paulson, S. K., & Breau, A. P. (2000). Characterization of metabolites of Celecoxib in rabbits by liquid chromatography/tandem mass spectrometry. *Journal of Mass Spectrometry* **35**, 1259-1270.

Zhang, Y., Shaffer, A., Portanova, J., Seibert, K., & Isakson, P. C. (1997). Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E-2 production. *Journal of Pharmacology and Experimental Therapeutics* **283**, 1069-1075.

Zhao, S. Z., Reynolds, M. W., Lefkowitz, J., Whelton, A., & Arellano, F. M. (2001). A comparison of renal-related adverse drug reactions between rofecoxib and celecoxib, based on the World Health Organization/Uppsala Monitoring Centre safety database. *Clinical Therapeutics* **23**, 1478-1491.

Appendix

Appendix Table 1: Table showing typical mean (\pm SEM) values of biochemical markers and ions in blood of the six horses

Parameters	SI units	Reference values	Pre administration Data are mean \pm SEM	Post administration Data are mean \pm SEM
RBC	$10^{12}/L$	8.0-12.4	9.19 ± 0.42	9.53 ± 0.57
Hb	g/dL	11-16	12.84 ± 0.93	13.14 ± 0.88
PCV	L/L	0.30-0.45	0.40 ± 0.02	0.41 ± 0.01
MCV	fL	34-58	43.46 ± 1.88	46.47 ± 1.81
MCH	pg	13.0-19.0	15.58 ± 0.66	16.33 ± 0.80
MCHC	g/dL	31-37	34.95 ± 1.09	35.31 ± 0.74
PLT	$10^9/L$	150-350	244.42 ± 14.18	244.85 ± 13.01
WBC	$10^9/L$	5-14	8.0 ± 0.23	7.81 ± 0.22
NEU	%	40-60	50.88 ± 1.35	49.60 ± 1.32
LYM	%	36-48	41.84 ± 2.28	40.02 ± 1.78
MONO	%	3-6	5.02 ± 0.15	4.81 ± 0.11
EOS	%	1-2	1.42 ± 0.19	1.61 ± 0.09
BASO	%	0-1	0.6 ± 0.06	0.67 ± 0.05
IRON	$\mu\text{mol/L}$	14-25	24.17 ± 1.42	24.08 ± 1.05
CK	U/L	120-300	197.67 ± 4.87	196.21 ± 5.0
LDH	U/L	250-500	247.18 ± 9.36	245.6 ± 10.68
AST	U/L	150-300	234.01 ± 10.15	231 ± 11.08
ALT	U/L	1-10	5.57 ± 0.36	6.42 ± 0.48
GGT	U/L	10-40	17 ± 1.13	18.42 ± 1.30
CREA	$\mu\text{mol/L}$	88-177	121 ± 10.17	122.57 ± 10.67
BUN	mmol/L	1.5-9.0	4.35 ± 0.39	4.61 ± 0.48
TP	g/L	55-85	69.71 ± 3.06	72 ± 2.83
ALB	g/L	30-70	32.28 ± 1.56	32.57 ± 0.94
MG	mmol/L	0.7-0.9	0.75 ± 0.04	0.75 ± 0.02
CA	mmol/L	2.5-3.5	3.05 ± 0.11	2.99 ± 0.10
PHOS	mmol/L	0.8-1.5	0.90 ± 0.02	0.99 ± 0.01
Na	mmol/L	126-158	140.71 ± 2.72	139 ± 2.42
K	mmol/L	2.6-6.2	4.58 ± 0.31	4.79 ± 0.15
Cl	mmol/L	95-119	101 ± 2.26	102.45 ± 1.68
Cu	$\mu\text{mol/L}$	9.0-14.0	15.24 ± 0.55	15.90 ± 0.62
Zn	$\mu\text{mol/L}$	6.0-10.0	12.85 ± 2.20	10.92 ± 1.83
SAA	mg/L	0-20	2.35 ± 0.32	2.24 ± 0.09

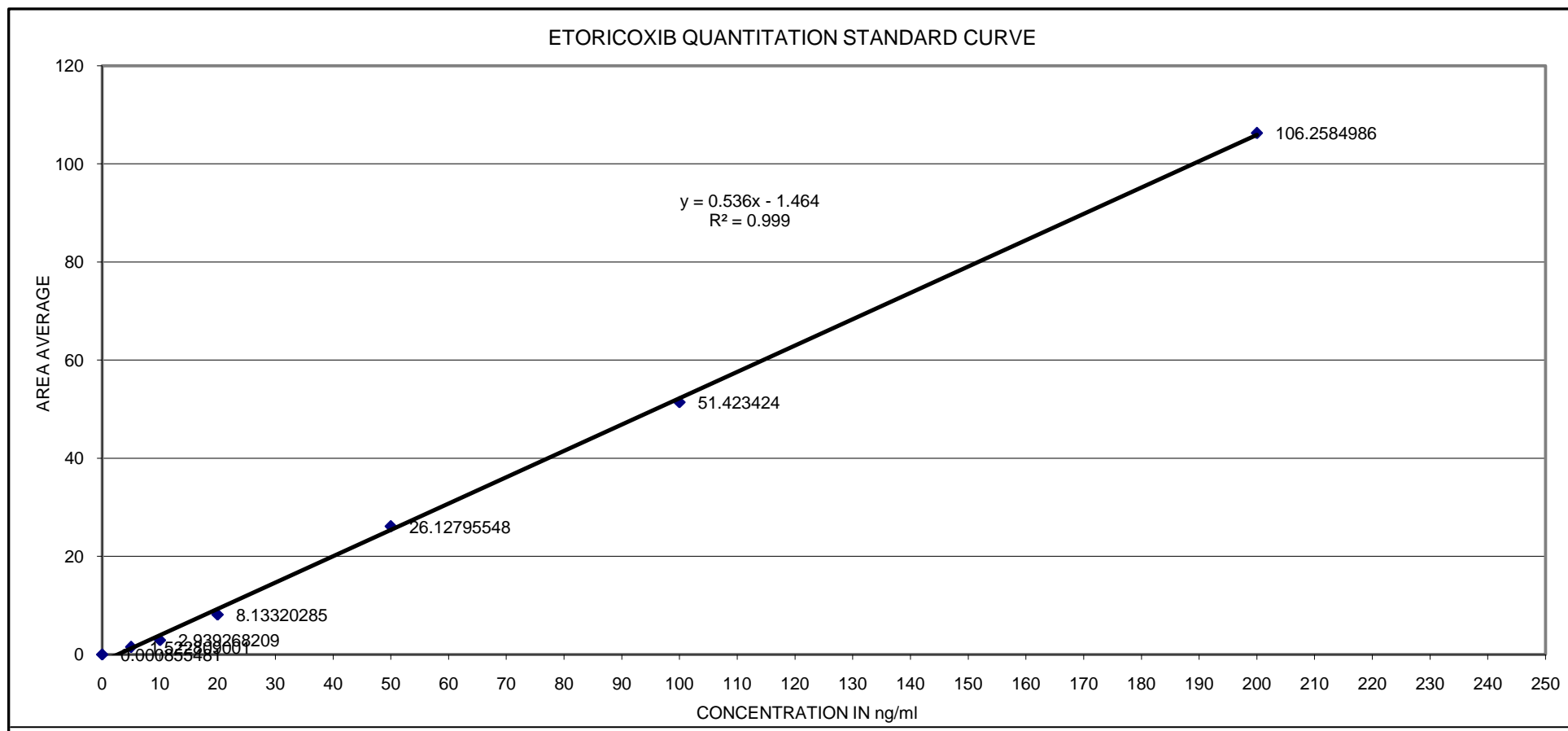
Appendix

Appendix Table 2: An example of values obtained for ETORICOXIB QUANTITATION - STANDARD CURVE

CALIBRANTS	ZALEPLON	ETORICOXIB	ETORICOXIB/ZALEPLON	AVERAGE
0 NG ETOR, 200 NG ZAL	1290391	2087	0.001617339	0.008554812
0 NG ETOR, 200 NG ZAL	280204	4341	0.015492284	
5 NG ETOR, 200 NG ZAL	579312	939528	1.621799652	1.522809001
5 NG ETOR, 200 NG ZAL	776309	1105323	1.423818351	
100 NG ETOR, 200 NG ZAL	1173948	2797686	2.383143035	2.939268209
10 NG ETOR, 200 NG ZAL	501127	1751636	3.495393383	
20 NG ETOR, 200 NG ZAL	438459	4089163	9.326215222	8.13320285
20 NG ETOR, 200 NG ZAL	871383	6047564	6.940190479	
50 NG ETOR, 200 NG ZAL	758684	17895758	23.5878943	26.12795548
50 NG ETOR, 200 NG ZAL	658774	18885744	28.66801665	
100 NG ETOR, 200 NG ZAL	376319	17044479	45.29263471	51.42342457
100 NG ETOR, 200 NG ZAL	815401	46929764	57.55421443	
200 NG ETOR, 200 NG ZAL	618765	52295054	84.51521014	106.2584986
200 NG ETOR, 200 NG ZAL	330136	42257998	128.0017871	

0	0.00085548
5	1.522809
10	2.93926821
20	8.13320285
50	26.1279555
100	51.423424
200	106.258499

Y INTERCEPT=	-1.464
SLOPE	0.536
CORR COEFF	0.999



Appendix – (Table - 3) Consolidated results for CELECOXIB administration to six horses at 2 mg/kg bwt.

Sample	Sample	WING BEAT		JOULA		DUTCH GOLD		POMPEI		AZIMUTH		SHANIKO				
No	Time (hrs) Wt >>	423	423	456	456	509	509	487	487	485	485	442	442	Time (hrs) << Wt	Average	SEM
1	0.00001	0	0	-10.524	-12.192	-14	-14	-7.5499	-14	0	0	-7.5	-6.4	0.00001	-7.18	1.703144
2	0.25	12.97	14.852	40.277	40.172	46.611	29.937	80.562	48.58	16.85479	2.018488	8.55348	5.913763	0.25	28.94	6.679112
3	0.5	19.827	43.793	501.81	509.34	400.82	466	486.09	503.73	265.1437	481.4303	97.1648	118.7666	0.5	324.49	57.9621
4	1	52.307	35.492	673.94	653.73	560.26	464.61	654.46	627.72	393.8124	396.5148	282.8444	299.5764	1	424.60	64.91445
5	2	149.78	178.68	883.84	847.96	1011.3	736.88	996.38	1011	805.1106	784.4536	522.9252	509.5475	2	703.15	86.98334
6	3	252.05	397.01	870.26	896.74	670.57	903.46	744.8	894.54	784.4536	694.3158	734.4885	584.1708	3	702.24	59.28721
7	4	205.49	219.77	815.66	756.01	870.9	703.69	804.77	601.4	755.9014	755.9014	885.9835	912.5046	4	690.67	68.89556
8	5	821.56	830.32	855	929.15	1761.2	1611.4	820.45	804.33	814.5736	803.5968	1042.678	1272.411	5	1030.56	97.24718
9	6	747.71	759.72	838.85	1028.7	1629	1621.6	635.58	729.33	430.9372	368.3718	1149.458	1327.513	6	938.90	121.3542
10	7	714.48	716.13	759.42	958.39	1379	1020.7	683.28	455.85	366.8217	457.2374	1094.391	1130.325	7	811.34	89.4634
11	8	754.3	724.43	750.34	727.74	1349.9	1209.6	899.35	566.43	119.9961	111.2574	567.8043	430.167	8	684.28	107.1964
12	24	206.01	242.18	687.2	589.75	832.05	758.86	606.78	570.16	271.8787	87.16986	189.4904	150.5762	24	432.67	76.68223
13	48	197.26	197.06	150.09	133.5	259.26	180.85	164.05	282.36	60.6667	99.88622	29.66495	38.51457	48	149.43	23.47225
14	72	26.433	16.967	27.397	14.961	65.556	43.44	65.7	52.735	-8.563749	3.164667	3.717035	-0.4575	72	25.92	7.425292
	96	2.5219	11.16	11.167	5.5749	12.175	8.8574	16.932	20.256	-22.37652	-23.03283	-5.906706	-4.36791	96	2.75	4.087119
	120	-1.4807	-4.0329	-5.3907	-8.9109	-8.8708	-8.3716	-2.384	-3.2586	-29.66314	-30.41502	-6.939791	-6.55222	120	-9.69	2.835781
	144			-11.761	-12.395	-10.938	-7.0221			-29.57899				144		2.532248

	P.kinetic	WING BEAT		JOULA		DUTCH GOLD		POMPEI		AZIMUTH		SHANIKO				
No	parameters													Average	Std Dev	Std Err
1	C ₍₀₎	221.53	235.45	704.89	755.22	874.76	782.83	702.42	618.64	362.79	334.57	464.75	391.62	537.46	228.13	19.01
2	k	0.05	-0.02	0.02	0.02	0.03	0.03	0.02	0.01	0.03	0.02	0.04	0.03	0.02	0.02	0.00
3	c max ug/ml	821.56	830.32	883.84	1028.65	1761.24	1621.64	996.38	1011.02	814.57	803.60	1149.46	1327.51	1157.87	323.58	26.96

4	t max hrs	5.00	5.00	2.00	6.00	5.00	6.00	2.00	2.00	3.00	4.00	5.00	4.00	4.09	1.51	0.13
5	t1/2 hrs	13.33	-41.89	39.38	32.75	26.61	22.96	36.66	103.47	23.98	33.03	17.91	19.84	15.52	31.99	2.67
6	AUC (total) ug/ml/hr	18884.09	19505.70	30310.34	27717.64	43998.49	38081.00	31049.46	29873.74	29873.74	29873.74	29873.74	29873.74	29927.56	6751.04	562.59
7	AUC(0- 24)ug/ml/hr	#####	#####	#####	#####	#####	#####	#####	#####	17348.32	5330.53	1884.63				

AUC	0.00001	0	0	0	0	0	0	0	0	0	0	0	0
TRAPE	0.25	1.62	1.86	3.72	3.50	4.08	1.99	9.13	4.32	2.11	0.25	0.13	0.12
	0.5	4.10	7.33	67.76	68.69	55.93	61.99	70.83	69.04	35.25	60.43	13.21	15.59
	1	18.03	19.82	293.94	290.77	240.27	232.65	285.14	282.86	164.74	219.49	95.00	104.59
	2	101.04	107.09	778.89	750.85	785.77	600.75	825.42	819.37	599.46	590.48	402.88	404.56
	3	200.92	287.85	877.05	872.35	840.92	820.17	870.59	952.78	794.78	739.38	628.71	546.86
	4	228.77	308.39	842.96	826.38	770.73	803.57	774.78	747.97	770.18	725.11	810.24	748.34
	5	513.52	525.04	835.33	842.58	1316.07	1157.57	812.61	702.87	785.24	779.75	964.33	1092.46
	6	784.63	795.02	846.93	978.90	1695.13	1616.55	728.02	766.83	622.76	585.98	1096.07	1299.96
	7	731.09	737.93	799.14	993.52	1504.01	1321.19	659.43	592.59	398.88	412.80	1121.92	1228.92
	8	734.39	720.28	754.88	843.07	1364.43	1115.19	791.32	511.14	243.41	284.25	831.10	780.25
	24	7682.43	7732.87	#####	#####	#####	#####	#####	9092.72	3135.00	1587.42	6058.36	4645.95
	48	4839.23	5270.84	#####	8679.08	#####	#####	9249.97	#####	3990.54	2244.67	2629.86	2269.09
	72	2684.36	2568.34	2129.87	1781.57	3897.80	2691.47	2756.99	4021.17	625.24	1236.61	400.58	456.68
	96	347.46	337.53	462.76	246.43	932.76	627.57	991.59	875.89	-371.28	-238.42	-26.28	-57.90
	120	12.49	85.52	69.31	-40.03	39.65	5.83	174.58	203.96	-624.48	-641.37	-154.16	-131.04
	144	-17.77	-48.40	-205.82	-255.67	-237.70	-184.72	-28.61	-39.10	-710.91	-364.98	-83.28	-78.63

													Mean	Std Dev	Std Err
AUC (Total) TRAPEZ	18884.09	19505.70	30310.34	27717.64	43998.49	38081.00	31049.46	29873.74	12167.58	9466.63	15052.40	13593.24	24141.69	10961.78	<u>3305.10</u>
AUC (0- 24)TRAPE	11000.55	11243.47	17600.92	17010.56	26032.60	23479.61	17876.34	14542.48	7551.80	5985.35	12021.95	10867.40	14601.09	6058.77	<u>1826.79</u>

Appendix – (Table - 4) Consolidated results for ETORICOXIB administration to six horses at 0.5 mg/kg bwt.

Sample	Sample	WINGBEAT		JOULU	DUTCH GOLD		POMPEI		AZIMUTH		SHANIKO					
No	Time (hrs) Wt >>	448		456	448		487		485		442		Time (hrs) << Wt	Average	sd	sem
1	0.00001	2.6	0	0	0	0	0	0	0	5.6	0	0	0.00001	0.75	3.417	0.986
2	0.25	32.419	26.763	130.828	3.460	4.673	29.431	44.371	50.792	37.384	124.483	122.7677	0.25	55.22	47.245	13.638
3	0.5	103.871	184.631	272.927	156.584	155.425	142.558	187.989	138.023	98.789	275.521	277.74582	0.5	181.28	80.151	23.138
4	0.75	345.507	460.678	403.782	375.780	332.829	337.551	401.831	283.951	224.375	514.248	537.639067	0.75	383.47	140.176	40.465
5	1	156.878	280.136	306.998	245.689	284.947	242.214	216.008	318.115	156.410	416.512	475.816316	1	281.79	121.917	35.194
6	2	150.565	154.432	302.799	219.845	264.220	217.119	251.331	325.151	396.696	482.170	458.133036	2	292.95	135.167	39.019
7	3	113.814	114.762	141.297	112.763	156.986	130.022	164.370	281.671	349.092	359.278	367.654395	3	208.34	117.192	33.831
8	4	110.909	105.179	179.678	59.995	43.491	83.056	111.911	263.986	321.882	285.984	264.578152	4	166.42	105.799	30.542
12	8	77.329	73.053	125.312	24.009	11.921	74.912	79.912	231.498	80.807	128.192	122.215662	8	93.56	61.190	17.664
13	24	21.053	13.180	21.593	18.887	13.191	20.420	20.420	203.186	58.673	50.355	49.6624688	24	44.60	53.368	15.406
14	48	4.415	6.080	11.301	10.197	9.042	0.294	0.294	17.505	18.519	20.203	20.6541623	48	10.77	7.246	2.092
	72	3.115	3.630	-4.409	0.013	-0.445	-3.530	-2.866	-10.914	6.386	-3.600	-3.5511932	72	-1.47	5.779	1.668
	96	2.982	2.132	-4.821	-0.395	-0.512	-5.105	-3.983	-15.666	5.907	-4.288	-4.2878885	96	-2.55	6.694	1.932
	120	1.100	0.780	-5.378	-0.567	-0.395	-3.750	-3.750	-14.643	5.829	-4.783	-4.7539044	120	-2.76	6.347	1.832

	P.kinetic	WING BEAT		JOULU	DUTCH GOLD		POMPEI		AZIMUTH		SHANIKO				
No	parameters												Average	Std Dev	Std Err
1	C ₍₀₎	132.91	150.35	243.02	90.01	89.29	177.60	215.79	241.41	179.18	327.57	328.54	197.79	82.95	<u>7.54</u>
2	k	0.07	0.07	0.10	0.05	0.06	0.08	0.09	-0.01	0.04	0.08	0.08	0.07	0.03	<u>0.00</u>
3	c max ug/ml	345.51	460.68	403.78	375.78	332.83	337.55	401.83	325.15	396.70	514.25	537.64	383.72	72.93	<u>6.63</u>
4	t max hrs	0.75	0.75	0.75	0.75	0.75	0.75	0.75	2.00	2.00	0.75	0.75	1.00	0.51	<u>0.05</u>
5	t1/2 hrs	9.75	9.75	6.93	14.07	12.53	8.71	7.88	-99.13	17.59	8.84	8.64	10.61	33.19	<u>3.02</u>

6	AUC (total) ug/ml/hr	704.10	816.33	1161.13	732.26	1427.31	1970.88	2219.23	2219.23	2219.23	2219.23	2219.23	1290.18	666.21	<u>60.56</u>
7	AUC(0-24)ug/ml/hr	698.47	811.60	1173.53	733.21	1425.97	1955.76	2169.20	1281.11	596.73	225.54				

AUC	0.00001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TRAPE	0.25	4.38	3.35	16.35	0.43	0.58	3.68	5.55	6.35	5.37	15.56	15.35
	0.5	17.04	26.42	50.47	20.01	20.01	21.50	29.05	23.60	17.02	50.00	50.06
	1	56.17	80.66	84.59	66.55	61.03	60.01	73.73	52.75	40.40	98.72	101.92
	2	62.80	92.60	88.85	77.68	77.22	72.47	77.23	75.26	47.60	116.35	126.68
	3	153.72	217.28	304.90	232.77	274.58	229.67	233.67	321.63	276.55	449.34	466.97
	4	132.19	134.60	222.05	166.30	210.60	173.57	207.85	303.41	372.89	420.72	412.89
	5	112.36	109.97	160.49	86.38	100.24	106.54	138.14	272.83	335.49	322.63	316.12
	6	94.12	89.12	152.49	42.00	110.82	315.94	383.65	990.97	805.38	828.35	773.59
	7	49.19	43.12	73.45	21.45	200.90	762.65	802.65	3477.47	1115.84	1428.37	1375.03
	8	12.73	9.63	16.45	14.54	266.80	248.57	248.57	2648.29	926.30	846.69	843.80
	24	3.76	4.85	3.45	5.10	103.17	-38.83	-30.87	79.10	298.85	199.24	205.24
	48	3.05	2.88	-4.62	-0.19	-11.48	-103.62	-82.19	-318.96	147.51	-94.65	-94.07
	72	2.04	1.46	-5.10	-0.48	-10.88	-106.26	-92.79	-363.71	140.83	-108.86	-108.50
	96	0.55	0.39	-2.69	-0.28	23.70	225.01	225.01	878.59	-349.72	287.00	285.23
	120	#VALUE!	0.00	0.00	#####	#####	#####	#####	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	144	#VALUE!	0.00	0.00	#####	#####	#####	#####	#VALUE!	#VALUE!	#VALUE!	#VALUE!

												Mean	Std Dev	Std Err
AUC (Total) TRAPEZ	704.10	816.33	1161.13	732.26	1427.31	1970.88	2219.23	8447.58	4180.32	4859.47	4770.31	2844.45	2451.54	<u>775.24</u>
AUC (0-24)TRAPE	698.47	811.60	1173.53	733.21	1425.97	1955.76	2169.20	8251.66	4241.70	4775.97	4687.65	2811.34	2398.98	<u>758.62</u>

Appendix – (Table - 5) Consolidated results for etoricoxib administration to six horses at 1mg/kg bwt.

Sample	Sample	WINGBEAT		JOULA		DUTCH GOLD		POMPEI		AZIMUTH		SHANIKO					
No	Time (hrs) Wt >>	448		456		448		487		485		442		Time (hrs) Wt <<	Average	SD	SEM
1	0.00001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	37.92	10.95
2	0.25	124.48	122.77	127.51	130.83	179.72	140.46	93.54	92.79	122.99	105.49	124.48	122.77	0.25	123.98	110.95	32.03
3	0.5	275.52	277.75	267.10	272.93	473.78	433.37	216.46	244.51	194.36	206.56	275.52	277.75	0.50	284.63	145.78	42.08
4	0.75	514.25	537.64	397.83	403.78	585.39	556.60	497.13	501.90	489.26	442.12	514.25	537.64	0.75	498.15	164.07	47.36
5	1	416.51	475.82	283.13	307.00	610.62	610.62	502.97	436.32	262.99	258.40	416.51	475.82	1.00	421.39	167.97	48.49
6	2	482.17	458.13	253.99	302.80	580.62	548.56	423.11	240.92	197.27	201.62	482.17	458.13	2.00	385.79	133.24	38.46
7	3	359.28	367.65	169.21	141.30	475.36	407.24	264.67	216.80	165.23	170.14	359.28	367.65	3.00	288.65	95.56	27.59
8	4	285.98	264.58	182.85	179.68	364.93	327.46	234.21	184.77	129.87	127.69	285.98	264.58	4.00	236.05	90.36	26.08
9	5	247.70	249.85	164.50	173.86	325.99	325.99	170.49	162.92	109.30	104.14	247.70	249.85	5.00	211.03	80.16	23.14
10	6	210.20	203.26	142.98	156.34	269.88	269.88	150.59	147.05	59.48	52.26	210.20	203.26	6.00	172.95	73.20	21.13
11	7	200.82	200.59	128.69	125.25	221.72	232.89	107.19	114.13	50.70	51.37	200.82	200.59	7.00	152.90	63.45	18.32
12	8	119.19	122.22	117.43	125.31	226.73	225.32	94.65	88.78	38.90	38.51	128.19	122.22	8.00	120.62	18.70	5.40
13	24	20.35	19.66	21.43	21.59	75.55	62.57	32.68	32.70	37.25	38.56	50.35	49.66	24.00	38.53	5.51	1.59
14	48	9.20	9.65	11.37	11.30	10.57	28.17	14.29	15.50	12.09	11.88	20.20	20.65	48.00	14.57	4.39	1.27
	72	-3.60	-3.55	-4.41	-4.41	-2.14	-1.39	-4.19	-3.48	-0.14	-0.14	-3.60	-3.55	72.00	-2.88	4.61	1.33
	96	-4.29	-4.29	-4.82	-4.82	-3.92	-4.22	-4.92	-4.84	-5.07	-5.07	-4.29	-4.29	96.00	-4.57	4.74	1.37
	120	-4.78	-4.75	-5.38	-5.38	-4.61	-4.92	-5.33	-5.26	-5.29	-5.29	-4.78	-4.75	120.00	-5.04	4.82	1.39
	144	-5.27	-5.27	-5.38	-5.38	-5.18	-5.08	-5.49	-5.58	-5.60	-5.60	-5.27	-5.27	144.00	-5.36	4.81	1.39
	168	-5.40	-5.40	-5.44	-3.82	-5.34	-5.43	-5.58	-5.58	-5.22	-5.22	-5.40	-5.40	168.00	-5.27	4.69	1.35
	192	-5.23	-5.21	-3.31	-3.04	-5.48	-5.30	-5.12	-4.87	-3.66	-3.66	-5.23	-5.21	192.00	-4.61	#DIV/0!	#DIV/0!

	P.kinetic	WING BEAT		JOULA		DUTCH GOLD,		POMPEI		AZIMUTH		SHANIKO				
No	parameters													Average	Std Dev	Std Err
1	C ₍₀₎	338.49	339.6	232.90	240.01	474.48	409.14	260.60	232.67	163.11	157.87	325.13	325.14	291.60	94.85	7.90
2	k	0.11	0.08	0.10	0.10	0.08	0.06	0.09	0.09	0.09	0.08	0.08	0.08	0.09	0.01	0.00
3	c max ug/ml	514.25	537.64	397.83	403.78	610.62	610.62	502.97	501.90	489.26	442.12	514.25	537.64	512.46	67.80	5.65
4	t max hrs	5.00	5.00	2.00	6.00	5.00	6.00	2.00	2.00	3.00	4.00	5.00	6.00	4.09	1.60	0.13
5	t1/2 hrs	6.19	8.72	6.98	6.93	9.12	11.86	7.48	7.87	8.11	8.38	8.84	8.64	8.30	1.44	0.12
6	AUC (total) ug/ml/hr	3778.28	3803.10	2953.29	3188.31	6523.35	6535.03	3507.93	3188.14	3188.14	3188.14	3188.14	3188.14	4184.68	1276.10	106.34
7	AUC(0-24)ug/ml/hr	2334.63	2337.11	1476.00	1535.64	3043.24	2888.93	1913.74	1593.47	2140.34	610.56	215.87				

AUC	0.00001												
TRAP E	0.25	15.56	15.35	15.94	16.35	22.46	17.56	11.69	11.60	15.37	13.19	15.56	15.35
	0.5	50.00	50.06	49.33	50.47	81.69	71.73	38.75	42.16	39.67	39.01	50.00	50.06
	1	98.72	101.92	83.12	84.59	132.40	123.75	89.20	93.30	85.45	81.09	98.72	101.92
	2	116.35	126.68	85.12	88.85	149.50	145.90	125.01	117.28	94.03	87.56	116.35	126.68
	3	449.34	466.97	268.56	304.90	595.62	579.59	463.04	338.62	230.13	230.01	449.34	466.97
	4	420.72	412.89	211.60	222.05	527.99	477.90	343.89	228.86	181.25	185.88	420.72	412.89
	5	322.63	316.12	176.03	160.49	420.15	367.35	249.44	200.79	147.55	148.91	322.63	316.12
	6	266.84	257.22	173.67	176.77	345.46	326.73	202.35	173.84	119.58	115.91	266.84	257.22
	7	228.95	226.56	153.74	165.10	297.94	297.94	160.54	154.98	84.39	78.20	228.95	226.56
	8	205.51	201.93	135.83	140.80	245.80	251.39	128.89	130.59	55.09	51.82	205.51	201.93
	24	160.01	161.41	123.06	125.28	224.23	229.10	100.92	101.45	44.80	44.94	164.51	161.41
	48	1116.37	1135.03	1110.86	1175.24	2418.28	2303.10	1018.64	971.78	609.21	616.57	1428.37	1375.03
	72	354.69	351.80	393.62	394.73	1033.46	1088.94	563.65	578.41	592.08	605.24	846.69	843.80

	96	67.24	73.24	83.56	82.70	101.14	321.41	121.19	144.29	143.44	140.89	199.24	205.24
	120	-94.65	-94.07	-110.76	-110.76	-72.78	-67.34	-109.29	-99.82	-62.46	-62.46	-94.65	-94.07
	144	-108.86	-108.50	-122.38	-122.38	-102.44	-109.72	-122.93	-121.14	-124.36	-124.36	-108.86	-108.50

														Mean	Std Dev	Std Err
AUC (Total)		3803	2953.2	3188.3	6523.3	6535.0	3507.9	3188.1	2298.6	2298.3	4614.1	4540.5		3935.76	1412.5	<u>425.8</u>
TRAPEZ	3778.28	.10	9	1	5	3	3	4	1	2	9	8			3	<u>9</u>
AUC (0-24)TRAPE		2337	1476.0	1535.6	3043.2	2888.9	1913.7	1593.4	1097.3	1076.5	2339.1	2337.1		1997.73	651.11	<u>196.3</u>
	2334.63	.11	0	4	4	3	4	7	1	1	3	1				<u>2</u>

Appendix – (Table - 6) Consolidated results for etoricoxib administration to six horses at 2 mg/kg bwt.

Sample	Sample	WINGBEAT	JOULA,	DUTCH GOLD	POMPEI	AZIMUTH	SHANIKO				
		448 KGS	456 KGS	509.5	487 KGS	485 KGS	442 KGS				
No	Time (hrs) Wt >>	448	456	448	487	485	442	Time (hrs) << Wt	Average	SD	SEM
1	0.00001	0	0	0	0	0	0	0.00001	0.00	74.34705	30.35206
2	0.25	191.212	200.798	139.202	87.217	186.257	204.331	0.25	168.17	239.4372	97.74982
3	0.5	590.458	588.119	409.520	522.146	655.777	725.952	0.5	582.00	374.9312	153.065
4	0.75	1040.899	1023.866	862.746	1105.219	832.887	905.688	0.75	961.88	346.5415	141.475
5	1	928.347	946.185	819.397	1013.289	891.509	838.942	1	906.28	318.0959	129.8621
6	2	706.791	641.762	689.997	1016.348	881.410	661.641	2	766.32	265.081	108.2189
7	3	531.774	563.917	587.870	870.718	661.131	642.351	3	642.96	252.8309	103.2178
8	4	393.422	353.517	500.698	857.789	488.193	511.558	4	517.53	158.2639	64.61096
12	8	362.734	329.560	474.951	465.820	402.059	355.820	8	398.49	42.76019	17.45677
13	24	136.527	108.097	76.808	125.188	93.940	92.535	24	105.52	13.39965	5.470385
14	48	21.668	22.722	48.774	34.922	21.129	20.368	48	28.26	7.166311	2.925634
	72	-2.774	-2.921	11.602	4.547	15.654	11.005	72	6.19	4.585939	1.872202
	96	-3.811	-3.706	8.359	1.188	3.308	0.743	96	1.01	4.286662	1.750022
	120	-4.505	-4.463	5.621	-1.216	-0.283	-0.946	120	-0.97	#DIV/0!	#DIV/0!

	P.kinetic	WINGBEAT	JOULA,	DUTCH GOLD,	POMPEI	AZIMUTH	SHANIKO			
No	parameters							Average	Std Dev	Std Err
1	C ₍₀₎	625.56	604.99	541.75	685.17	661.93	651.15	628.42	50.85	8.48
2	k	0.06	0.07	0.05	0.06	0.07	0.08	0.06	0.01	0.00

3	c max ug/ml	1040.90	1023.87	862.75	1105.22	891.51	905.69	975.84	97.98	<u>16.33</u>
4	t max hrs	5.00	2.00	5.00	2.00	3.00	5.00	3.67	<u>1.51</u>	<u>0.25</u>
5	t1/2 hrs	11.56	9.96	13.01	11.60	9.27	9.07	11.51	<u>1.56</u>	<u>0.26</u>
6	AUC (total) ug/ml/hr	3177.68	3053.05	3269.67	13289.55	10614.12	9791.31	5697.49	4566.78	<u>761.13</u>
7	AUC(0-24)ug/ml/hr	3187.39	3062.68	3249.89	13148.12	5662.02	#REF!			

AUC	0.00001	0.00	0.00	0.00	0.00	0.00	0.00
TRAPE	0.25	23.90	25.10	17.40	10.90	23.28	25.54
	0.5	97.71	98.61	68.59	76.17	105.25	116.29
	1	203.92	201.50	159.03	203.42	186.08	203.96
	2	246.16	246.26	210.27	264.81	215.55	218.08
	3	817.57	793.97	754.70	1014.82	886.46	750.29
	4	619.28	602.84	638.93	943.53	771.27	652.00
	5	462.60	458.72	544.28	864.25	574.66	576.95
	6	378.08	341.54	487.82	2647.22	1780.51	1734.76
	7	249.63	218.83	275.88	4728.06	3967.99	3586.84
	8	79.10	65.41	62.79	1921.31	1380.83	1354.84
	24	9.45	9.90	30.19	473.62	441.40	376.47
	48	-3.29	-3.31	9.98	68.82	227.55	140.98
	72	-4.16	-4.08	6.99	-0.33	36.30	-2.43
	96	-2.25	-2.23	2.81	72.95	16.98	56.75
	120	0	0	0	0	0	0
	144	0	0	0	0	0	0

								Mean	Std Dev	Std Err
AUC (Total) TRAPEZ	3177.68	3053.05	3269.67	13289.55	10614.12	9791.31		7199.23	4566.78	<u>2042.33</u>
AUC (0-24)TRAPE	3187.39	3062.68	3249.89	13148.12	10333.28	9596.01		7096.23	4465.33	<u>1996.95</u>

